CAR co-operates with Filamin A to regulate epithelial cell adhesion and migration

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CAR Co-operates with Filamin A to Regulate Epithelial Cell Adhesion and Migration

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अज्ञानतिमिरान्धस्य ज्ञानाञ्जनशलाकया ।

चक्षुरुन्मीलितं येन तस्मै श्रीगुरवे नमः ।।

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Abstract

Coxsackie and Adenovirus receptor (CAR) is a member of the JAM family of adhesion receptors. CAR is located at both tight and adherens junctions between epithelial cells, where it assembles adhesive contacts through homodimerisation in trans. However, the recruitment, binding partners and signaling effects of CAR at cell junctions and how CAR co-ordinates cell:cell and cell:matrix adhesions remain poorly understood. We have identified Filamin A (FLNa), an F-actin cross-linker, as a novel CAR-binding protein. Our biochemical analysis revealed that the cytotail of CAR phosphorylated at Ser293/Thr290 directly binds to FLNa rod1 and actin-binding domains. FLNa is known to bind β1-integrins and negatively regulate integrin activation, which in turn impacts the assembly of focal adhesions. Our data demonstrates that phosphorylation of CAR also impacts on activation of integrins. Filamin-CAR interaction may, thus, control the localized activation of β 1-integrins and focal adhesion assembly. Furthermore, overexpressing CAR increases epithelial cell migration rates and unphosphorylated CAR promotes more stable cell:cell adhesion. Taken together, our data reveals the importance of CAR as a molecular switch in controlling the cross-talk between cell:cell and cell:matrix contacts. This paves way for future studies in adhesion and migratory signaling under homeostatic and inflammatory conditions in epithelial cells.

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List of Abbreviations

AACAR	S293A/T290A cytoplasmic mutants of CAR
Ad	Adenovirus
AJ	Adherens Junction
CAR	Coxsackie and Adenovirus Receptor
CT	Cytoplasmic Domain
C-terminal	Carboxy-terminal
CVB	Coxsackievirus group B
DDCAR	S293D/T290D cytoplasmic mutants of CAR
ECM	Extracellular Matrix
FA	Focal Adhesions
FAK	Focal Adhesion Kinase
F-actin	Filamentous Actin
FLCAR-GFP	GFP tagged Full Length Coxsackie and Adenovirus Receptor
FLNa	Filamin A
FRET	Fluorescence (Förster) Resonance Energy Transfer
GFP	Green Fluorescent Protein
GST	Glutathione S-Transferase
HBEC	Human Bronchial Epithelial Cell
HSC 70	Heat Shock Complex
IF	Immunofluorescence
Ig	Immunoglobulin
IP	Immunoprecipitation
JAM	Junctional Adhesion Molecule
MAPK	Mitogen-activated protein kinase
N-terminal	Amino-terminal
PBS	Phosphate Buffered Saline

PKC	Protein Kinase C
SDS- PAGE	Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis
Ser	Serine
Thr	Threonine
TJ	Tight Junction
Tyr	Tyrosine
WB	Western Blotting
16HBE	Human Bronchial Epithelial Cells

1. Introduction

1.1. **Epithelial Cells: The Biological Gatekeepers**

Epithelia are the biological gatekeepers that border all biological compartments and regulate the trafficking of ions, solutes, fluid and immune cells. They simultaneously protect underlying tissues and allow selective passage of molecules and cells. The latter is mediated by cell adhesion complexes which are known to control cell proliferation during tissue development, would healing and carcinogenesis and are therefore important in maintaining epithelial homeostasis (Farkas et al 2012). Cell:cell junctions or adhesion complexes are specialised areas in the plasma membrane that occur at points of cell:cell contact and are essential in epithelial cells. Based on their functions, cell:cell junctions can be broadly classified as (i) occluding or tight junctions (TJ), which are belts of proteins that seal the extracellular space between cells; (ii) anchoring or adherens junctions (AJ), which mechanically attach cytoskeletons in one cell to that of adjacent cells or in the case of focal adhesions (FA), to the extracellular matrix (ECM); and (iii) communicating or gap junctions which mediate passages of signals, either chemical or mechanical, between an interacting cell and its partner (Alberts et al 2002). These structures (Figure 1.1) may be further sub-classified as summarised in Table 1.1.

Table 1.1. ***Classification of epithelial cell junctions based on their functions***

Type	Function	Major proteins involved
OCCLUDING JUNCTION		
Tight Junctions	Seal neighbouring cells together in an epithelial sheet to prevent leakage of molecules between them	Transmembrane proteins: claudins and occludins; intracellular peripheral membrane protein: zonula occludens
ANCHORING JUNCTION		
Actin filament attachment sites		
Adherens Junctions	Join actin filaments of neighbouring cells	E-cadherins and anchoring proteins (catenins, vinculin & α-actinin)
Focal Adhesions	Link intracellular actin to extracellular matrix	Integrins, which indirectly bind actin filaments via intracellular anchor proteins (vinculin, talin, α-actinin & filamin)
Intermediate filament (Keratin) attachment sites		
Desmosomes	Join intracellular keratin of neighbouring cells	Anchor proteins: plakoglobin & desmoplakin; adhesion proteins: desmoglein & desmocollin
Hemidesmosomes	Anchor intermediate filaments to basal lamina	Integrins bind to intermediate filaments via anchor protein (plectin)
COMMUNICATING JUNCTION		
Gap Junctions	Allow passage of small molecules	Connexins

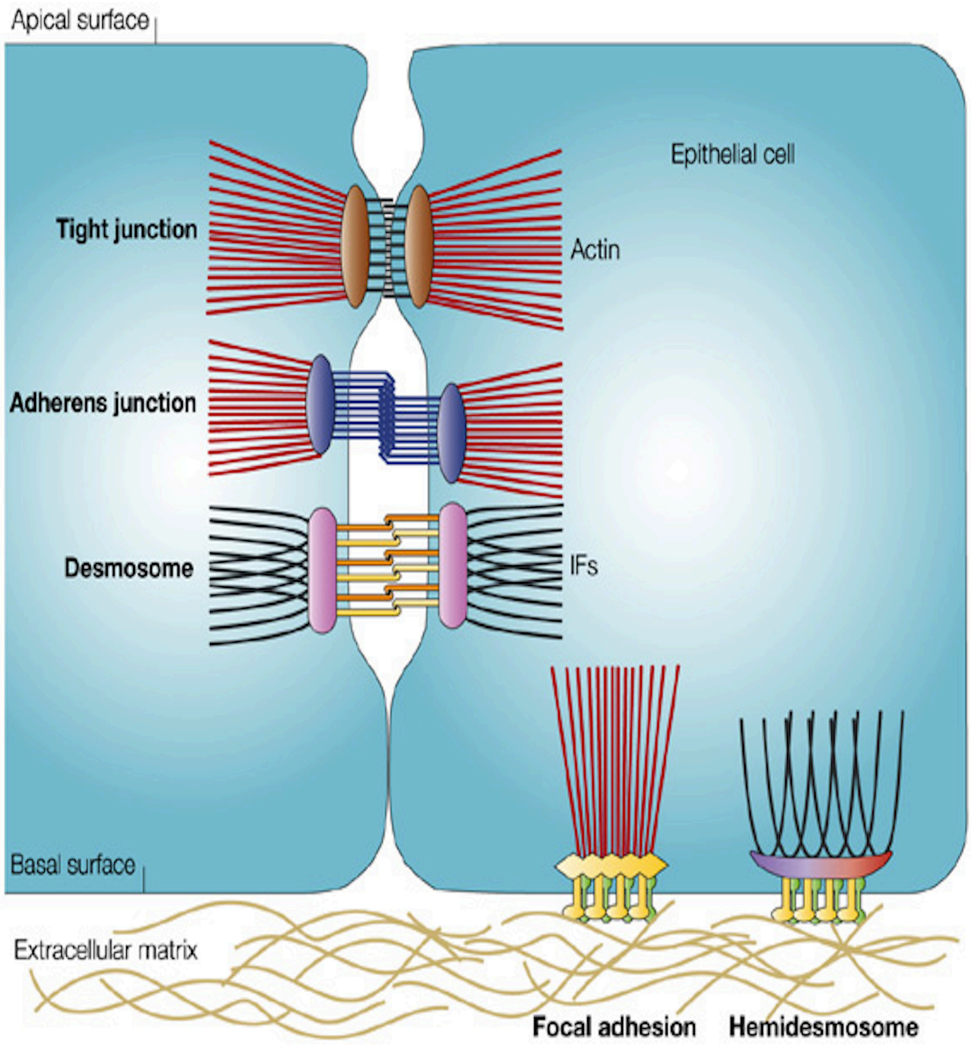


Fig 1.1. **Cell-adhesion complexes in mammals.** Epithelial cells are especially abundant in cell-adhesion complexes, which enable a tightly regulated cross talk between cells and their microenvironment. Five types of adhesion complexes are illustrated in this figure. The table above summarises their functions and protein compositions. (Image Source: Jefferson *et al* 2004)

1.2. **Maintenance of Airway Epithelia**

The airway epithelia plays a central role in responding to pulmonary inflammation and regulating innate and adaptive response (reviewed in Hiemstra et al 2015). The airway or lung epithelial cells located between the external and internal milieu, are insulted by a variety of allergens, pathogens and inhaled gases. Survival and maintenance of an integrated layer of epithelial cells is crucial and dependent on effective communication between cells and its extracellular environment through cell:cell and cell:matrix adhesive structures. These structures orchestrate well choreographed signaling events either through molecular handshakes between adhesive molecules present in the AJ and TJ or in response the pull of a cell:matrix adhesion called focal adhesions (FA). Since they are connected to the cell cytoskeleton through adaptor proteins, signals received are then conveyed to the cell interior.

1.2.1. *Cell:matrix adhesions*

Cell survival and cell cycle progression largely depend on cell:substratum adhesion and the subsequent mechanical stimulation inflicted from their attachment to the substratum or extracellular matrix (Huang et al 1996; Kim et al 2008; Chen et al 2011). Cell:matrix adhesion components include focal adhesion proteins and signalling molecules that regulate cell behaviour as summarized in Table 1.3

Focal adhesions are the physical link between the basolateral surface of the cell and underlying ECM regulated by Rho activity (Ridley, & Hall 1992). These integrin-receptor based adhesions are the signalling hubs and mechanosensors of cells and key mediators of cell:matrix communication (Chrzanowska-Wodnicka, & Burridge

1996) . Small focal complexes formed at the leading edges of cells in migration mature into stable focal adhesions (FA), which are predominantly localized centrally at the base of the cell but are also found at the periphery (Hynes 1992; Wozniak et al 2004). Focal adhesion kinase (FAK) is a key signalling protein that localizes to FA (Wozniak et al 2004) along with other kinases (e.g., Src), signalling scaffolds or adaptor proteins (e.g., paxillin, p130Cas, Crk) and anchorage/F-actin binding proteins (e.g., talin and vinculin). The scaffold proteins provide a platform for all the components of FA to interact and form complexes, which in turn tune cell morphology and behaviour, while the anchorage proteins link ECM to the actin cytoskeleton as well as providing additional hubs for signalling protein recruitment.

The coupling of 'inside-out' signalling and integrin-ligand binding, which facilitates 'outside-in' signalling, regulate the assembly of focal adhesions. Hence, it is a bidirectional phenomenon (Chrzanowska-Wodnicka, & Burridge 1996). Numerous structural components, such as α-actinin, filamin and talin, link integrin to the actin cytoskeleton (Wozniak et al 2004). While α-actinin is mostly found at newly assembling focal complexes, filamin binds to integrins at mature focal adhesions and inhibits integrin activation and thus cell migration (Calderwood et al 2001). Filamin is also known to strengthen adhesions (Giannone et al 2003), thereby, stabilizing cell:matrix interactions.

Table.1.2. ***Summary of cell behaviours regulated by key cell:matrix adhesion components.*** The table lists focal adhesion proteins (top panel) or small GTPases (bottom panel) and their roles in focal adhesion dynamics and different cell behaviours. Legend: +, protein has known positive role; −, protein has known negative regulation; +/−, protein has positive or negative role; ?, role is implicated (Wozniak et al 2004).

Protein	Integrin avidity	FA turnover	Cell migration	Mechano-sensing	Proliferation
FAK	+/−	+	+	+	+
Src	+/−	+	+	+	+
p130Cas	+/−		+		+
Crk	+/−		+		
Paxillin			+/−		
Talin	+	?		+	
Vinculin		?	+	+	
H-Ras	−	+	+		+
R-Ras	+	−	+/−		
Cdc42		+	+		+
Rac		+	+		+
Rho		−	+/−	+	+
Rap	+				

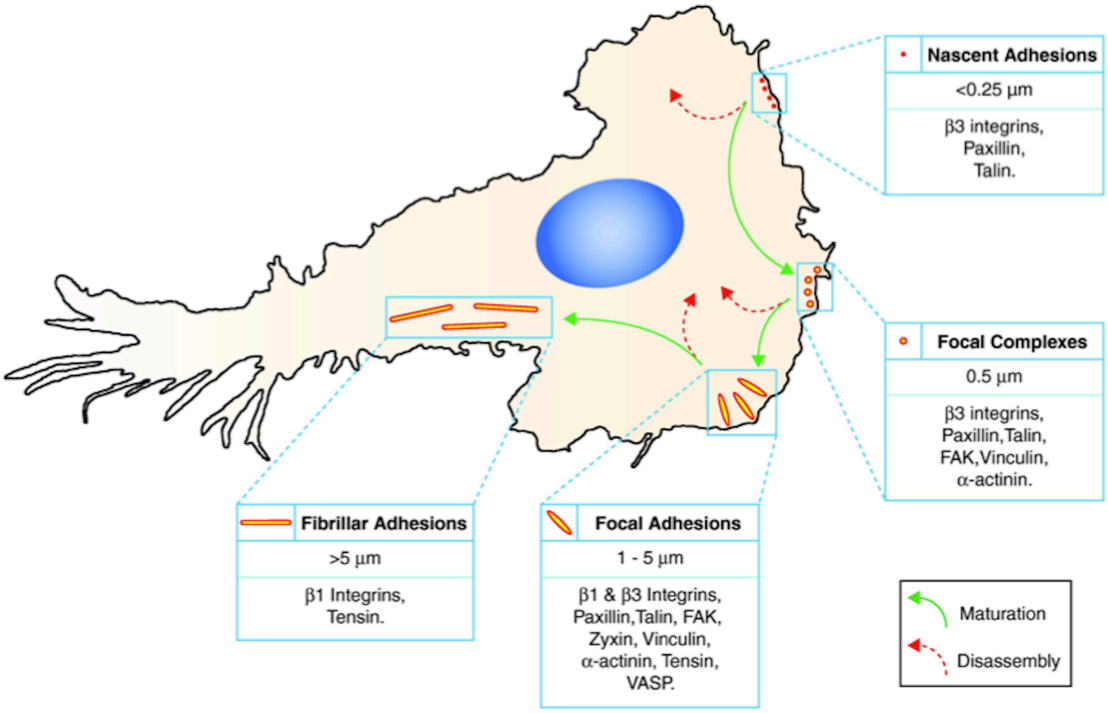


Fig.1.2. **Integrin based adhesions.** Based on the hierarchy of their maturation (green arrows) and disassembly (red arrows) they maybe classified as nascent adhesion (NA), focal complex (FC), focal adhesion (FA) and fibrillar adhesion (FB). Typical sizes and known proteins that localise to these adhesions are shown. (Image Source: Scales, & Parsons 2011)

1.2.1.1. *Integrins*

The largest and most predominant class of transmembrane ECM receptors, integrin plays a key role in physiological processes such as embryogenesis, wound healing, immune defence and in pathological processes such as cancer and autoimmune disease (Alberts et al 2002). Two non-covalently associated transmembrane glycoprotein subunits (α and β) make up the integrin receptor. They are known to exist in 24 different cell-specific combinations in mammals (Hynes 2002). Activation of integrins through the binding of β tail to intracellular proteins such as talin and kindlins leads to conformational change in the extracellular region thus permitting interactions with the ECM and other extracellular ligands (Hynes 2002).

Integrins modulates bidirectional signaling between the cell and extracellular matrix (Hu 2013). They bind ligands on their extracellular domain and facilitate 'outside in' signaling, ie: relay external signals to the cell. Through their cytoplasmic domain they interact with various intracellular proteins via mediators such as FAK and MAPK to convey signals from the cell to the matrix, thus, facilitating 'inside out signalling' (Boudreau, & Jones 1999). Therefore, integrins open a two way channel to mediate effective communication of the cell with its surrounding environment (Hu 2013). However, integrins do not have any direct catalytic activity and therefore rely on recruitment of numerous adaptor proteins, kinases and phosphatases to signalling plaques at the plasma membrane known as focal adhesions. Through recruitment of these proteins, such as vinculin, talin and paxillin, integrins can also connect indirectly to the actin cytoskeleton to allow cell shape changes in response to extracellular cues. Integrins therefore play a crucial role in organism development, cell adhesion, migration and proliferation, in addition to actively controlling cell architecture (Hynes 1992; Scales, & Parsons 2011).

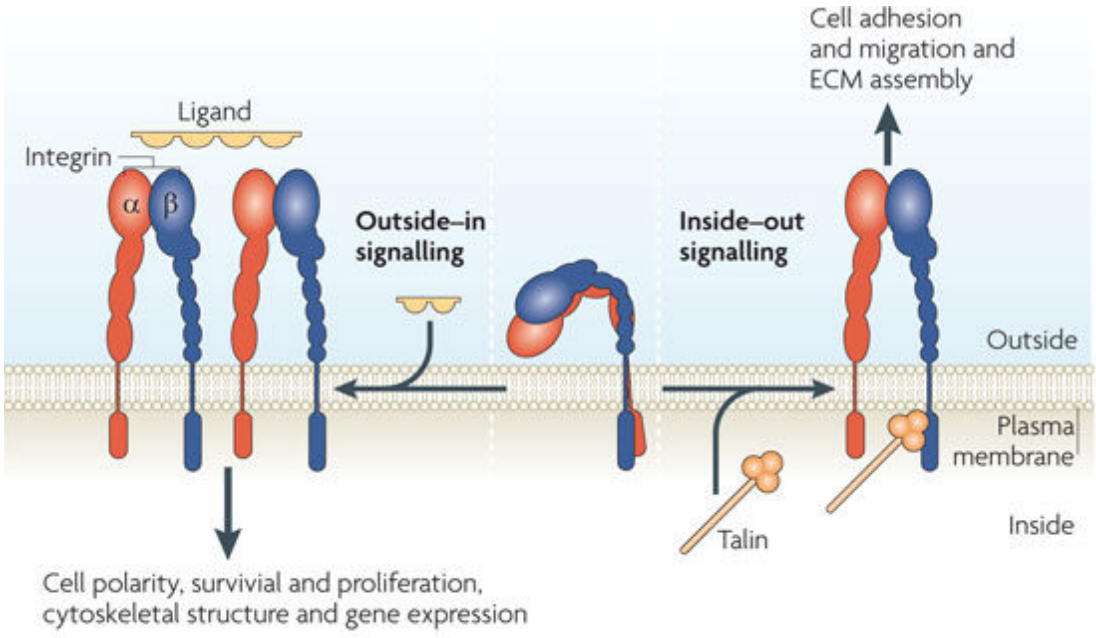


Fig.1.3. **Integrin structure & bidirectional signalling.** Integrins are heterodimeric adhesion receptors composed of an α- and β-subunit. A helical transmembrane domain separates the large globular N-terminal domain from short unstructured cytoplasmic domain on each subunit. Both dimerization and ligand binding to the N-termini of the subunits are cation dependent events. The β-subunit when linked to the cytoskeleton via adaptor proteins allows the cell to respond to pericellular changes. (Source: Shattil et al 2010)

In order to effectively control adhesion assembly in the correct spatial manner integrins are in turn controlled through classes of activator and inactivator proteins that bind directly to the C-terminal domain of the β-integrin subunits (Alberts et al 2002). The integrin activators are talin and kindlin family proteins that bind directly to NPXY amino acid sequences within the β-integrin subunits (Tadokoro et al 2003; Harburger et al 2009). Binding of these proteins acts to induce a conformational change in the extracellular domain of the integrin and enable it to bind to ECM ligands. Conversely, integrins are inactivated through binding to either filamin, integrin cytoplasmic associated protein (ICAP-1) or sharpin (Chang et al 1997; Loo et

al 1998; Rantala et al 2011). The way in which these proteins act to inhibit integrin activation remains controversial, but studies have shown a clear role for each in regulating different subclasses of integrin function in a range of cell types. Moreover, not much is currently known about how these proteins co-ordinate with one another to dictate cell adhesion under different environmental conditions, or settings.

1.2.1.2. *F-Actin*

Filamentous actin (F-actin) is vital in the regulation of cell shape and movement both of which are essential to maintaining a healthy intact epithelial monolayer (Pollard et al 2009). The polymeric chains of G-actin monomers form polarised filaments in an ATP-dependent manner (Korn et al 1987). They comprise of a 'pointed' end, which is the origin of a growing filament and a fast growing 'barbed' end, where new monomers are added to the growing filament (Pollard et al 2003). Actin filaments laterally associate to form actin cables or bundles aided by cross-linkers such as α-actinin, filamin (section 1.5) and myosin. When two actin filaments have their barbed ends in the same direction, they are termed parallel; when one filament's barbed end and the other filaments pointed end are in the same direction, they are called antiparallel bundles. Actin filaments form a number of different cell structures as described below:

- Lamellipodia: These are broad 'sheet-like' protrusions characterised by short actin cables arranged in a loose meshwork at the cell edge. These structures are associated with sensing of the external environment and crucial to driving cell migration (reviewed in Krause, & Gautreau 2014)

- Filopodia: These are thin long, unbranched, parallel actin bundles extending from the cell membranes, whose elongation is mediated by formin proteins (Mellor 2010). They are associated with proteins such as fascin and tropomyosin, and are involved in probing their local environment to mediate cell invasion (Vignjevic et al 2006; Mattila et al 2008; Creed et al 2011).
- Stress fibres: These are thick antiparallel actin bundles decorated with myosin II, which confers them with contractility. They are mostly found at the base of the cell or along the rear end of a migrating cell, and terminate in focal adhesions (Hotulainen et al 2006).
- Arcs: These are large actin bundles found on the dorsal and lateral sides of the cell. They confer transverse structural support to the cells and usually terminate in focal adhesions (Hotulainen et al 2006).

The formation and maintenance of these structures largely depends on (i) actin polymerisation at the barbed ends promoted by proteins called nucleators, for example formins (Zigmond 2004); and (ii) capping, a process by which actin elongation is terminated by capping proteins, for example gelsolin (Gremm, & Wegner 2000). Actin structures are formed, turned over and rearranged in response to external cues and cell processes such as cell division and cell migration, all of which are mediated by signalling proteins such as Rho-GTPases (reviewed in Sit, & Manser 2011).

1.2.2. *Tight Junctions*

Specialized sealed structures formed by the outer leaflets of plasma membrane on adjacent cells at the apical and basolateral regions are referred to as tight junctions (TJs) or *zonula occludens* (Bazzoni, & Dejana 2004*)*. Since they mediate paracellular

permeability by regulating the diffusion of solutes across intercellular spaces, and maintain cell polarity by retaining membrane molecules at specific apical and basolateral domains, they are said to have “barrier” and “fence” function, respectively (Bazzoni, & Dejana 2004). TJs are composed of integral membrane proteins: occludins, claudins and junctional adhesion molecules (JAM) and intracellular proteins ZO-1, ZO-2 and ZO-3.

The JAM proteins have myriad functions including formation of cell:cell adhesion, maintaining barrier function, angiogenesis, leukocyte transmigration, platelet activation and reovirus binding (Huber et al 2000; Garrido-Urbani et al 2014). They comprise two extracellular Ig-like domains, a hydrophobic transmembrane domain and cytoplasmic tail of variable length containing a PDZ domain. The “classic” proteins of the JAM family comprise a distinct protein group, which includes JAM-A, JAM-B and JAM-C. Proteins such as Coxsackie and Adenovirus Receptor (CAR), Endothelial Selective Adhesion Molecule (ESAM), JAM-L and JAM-4, which have a longer cytoplasmic tails, are categorised into a separate subfamily. Through their extracellular domains, JAMs can form homo and heterodimerisation *in trans*. JAMs interact with a number of adaptors and signalling proteins through the PDZ domain in the C-terminus (Garrido-Urbani et al 2014). JAM-C and JAM-L have been shown to interact with CAR (Garrido-Urbani et al 2014). JAM-L expressed on the surface of T-cells or neutrophils, bind CAR on epithelial cells to activate signalling pathways including the phosphoinositide-3-kinase (PI-3-K) pathway, resulting in cell proliferation and cytokine production (Zen et al 2005; Verdino et al 2010). The interaction of members of the JAM family with CAR is thought to have a regulatory role in controlling local immune responses.

The ZO proteins interact directly or indirectly with a wide range of partners; such as regulators of cell proliferation, signaling molecules (including the GTPase Ras and Rab family members), PDZ- containing proteins (eg: MUPP1), and cytoskeletal-associated components (eg: myosin II) (reviewed in, Fanning, & Anderson 1999). Hence, ZO is well known to assemble molecular complexes at TJ which are essential to mediate its "barrier' and "fence" functions (Fanning, & Anderson 1999).

1.2.3. *Adherens Junctions*

Located just below the tight junctions are continuous bands of E-cadherin receptors that form the basis of the the adherens junction (AJ), which connect lateral membranes on neighbouring cells. The homophilic interactions of the extracellular domains of E-cadherin facilitate dynamic adhesive contacts between epithelial cells (Gumbiner et al 1988). Upon binding three extracellular Ca^{2+} ions, the extracellular domain changes conformation and rigidifies to facilitate *trans* dimerization (Pokutta et al 1994; Nagar et al 1996; Pertz et al 1999). The affinity to bind the Ca^{2+} ions varies suggesting that jucntional calcium ion levels can influence E-cadherin stability and signalling to the cell interior (Chitaev, & Troyanovsky 1998; Prasad, & Pedigo 2005). Its cytoplasmic domain interacts with a number of intracellular proteins including β-catenin and p120 catenin which mediate its anchorage to the actin filaments through anchoring proteins, e.g. α-catenin and vinculin (Bershadsky 2004; Yonemura et al 2010) and influence stability of AJ (Ireton et al 2002; Thoreson et al 2000), respectively. The association of E-cadherin-catenin complex to actin plays a major role is determining cell shape and stability in addition to relaying intercellular and extracellular signals, which is essential to maintain epithelial integrity during

tissue homeostasis and remodeling (Baum, & Georgiou 2011). The establishment of stable AJ is regulated by a number of Rho family GTPases, Rac and Cdc42 (Braga 2002). GTPases are involved both in regulating AJ dynamics and positioning E-Cadherin at junctions as well as cell polarity and structure (Baum, & Georgiou 2011). The calcium independent adhesion molecule nectin is also found at AJ. It is connected to the actin cytoskeleton through PDZ-containing afadin protein (Takahashi et al 1999; Takai et al 2003). Together these AJ molecules work towards mediating effective communication between cells and maintain cell polarity.

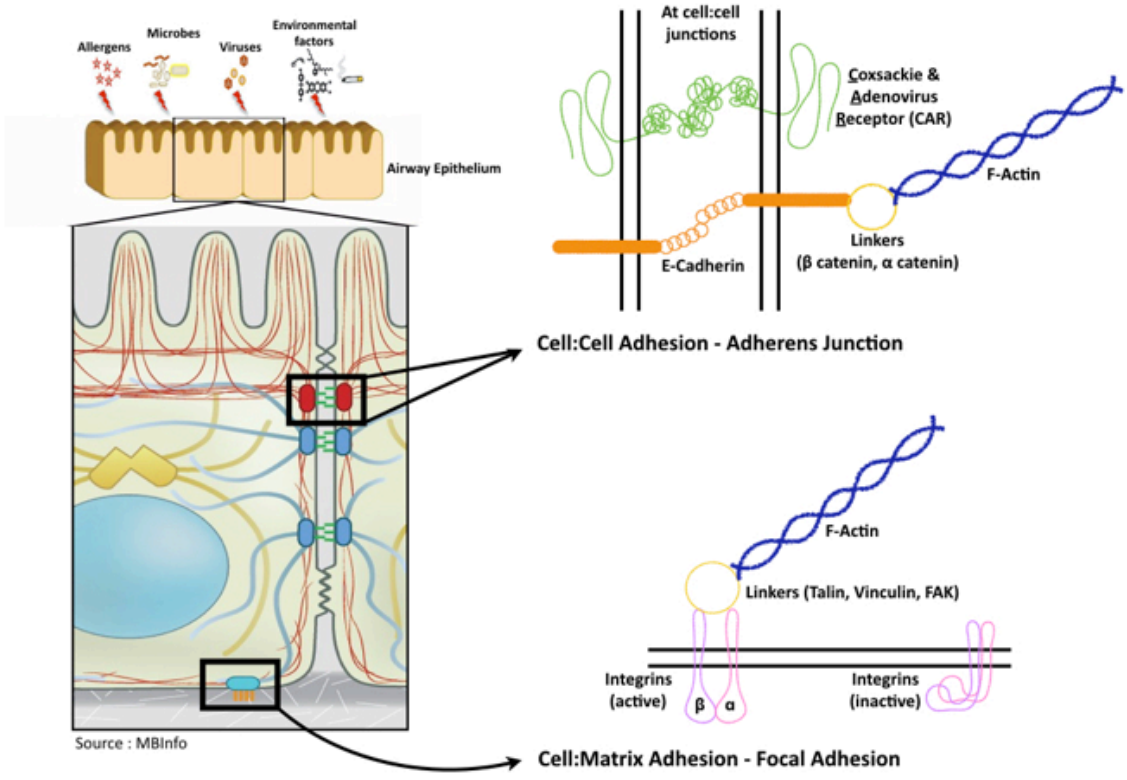


Fig.1.4. **Cell adhesive structures.** Schematic diagram showing the components of cell:cell adhestion (adherens junction) and cell:matrix adhesion (focal adhesions).

1.2.3.1 *Actin cytoskeleton in controlling cell:cell junctions*

In addition to physical linkages between adjacent cells and the ECM, structural mechanics is also vital to maintain stability of the epithelial layer. Focal adhesion components, vinculin and talin are known to directly or indirectly bind actin (Horwitz et al 1986; Hemmings et al 1996; Critchley 2009). Vinculin also indirectly influences actin filament nucleation in lamellipodia (DeMali et al 2002). Actin binding proteins, α-actinin and filamin directly bind to integrins (Otey et al 1990; Pfaff et al 1998). Furthermore, β-catenin links E-cadherins at the AJ to the circumferential actomyosin filaments via α-catenin, which directly regulate actin dynamics (Gates, & Peifer 2005). These elaborate interlinking of structural and adhesion proteins mediate extensive communication that are necessary to regulate the coupling between cell adhesion and protrusion followed by subsequent cell migration. Therefore, cell:cell and cell:matrix adhesions are essential to maintain an intact polarized epithelial layer (Vareille et al 2011).

1.3. **Coxsackie and Adenovirus Receptor (CAR)**

Originally identified as a docking receptor for group B coxsackievirus (CVB) and adenovirus serotypes (Ad), human coxsackie and adenovirus receptor (hCAR or CAR) is a 46 kDa class I membrane glycoprotein, which localises to tight junctions and adherens junctions (Cohen et al 2001; Hussain et al 2011). It belongs to the Cortical Thymocyte marker in Xenopus, CTX-subfamily of the immunoglobulin superfamily (IgSF), which includes Juctional Adhesion Molecule, A, -B, -C (Chrétien et al 1998; Martìn-Padura et al 1998; Palmeri et al 2000; Arrate et al 2001). CAR is a 360 amino acid transmembrane protein with a short leader sequence and two disulphide-linked loops. Its helical membrane-spanning domain separates a 216 amino acid

extracellular domain from a 107 amino acid intracellular domain (Philipson, & Pettersson 2004; Coyne, & Bergelson 2005). While the extracellular N-terminal domain of CAR is a high affinity receptor of adenovirus serotypes (Bergelson et al 1997), its C-terminal cytoplasmic tail is crucial for cell adhesion and growth modulatory functions (Excoffon et al 2010). Sites for phosphorylation and furin cleavage, palmitylation and PDZ-domain recognising hydrophobic peptide motif are present on the C-terminal domain (Xie et al 2006; van't Hof, & Crystal 2002; Coyne, & Bergelson 2005). Binding of the latter to the cyto-tail of CAR has been proposed to regulate trans-epithelial resistance *in vivo* and localisation of CAR within adherens junction *in vitro* (Excoffon et al 2004; Raschperger et al 2006). Two immunoglobulin (Ig)-like domains are present at the N-terminus, namely, D1 and D2. The CVB and Ad serotypes bind to specific sites on the homodimerised D1 domain. The residues involved in D1 dimerisation are highly conserved in evolution and, therefore, thought to be biologically important (Coyne, & Bergelson 2005). Since CAR mediates homotypic cell adhesion (Cohen et al 2001), the D1 dimerisation may shed some light on CAR's adhesive property. The structure of CAR is as shown in Figure 1. 3.

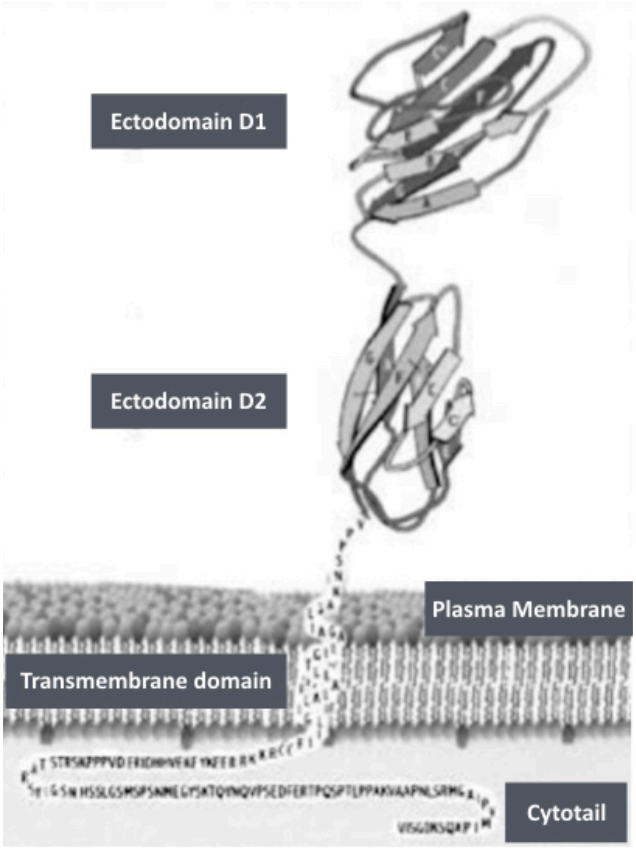
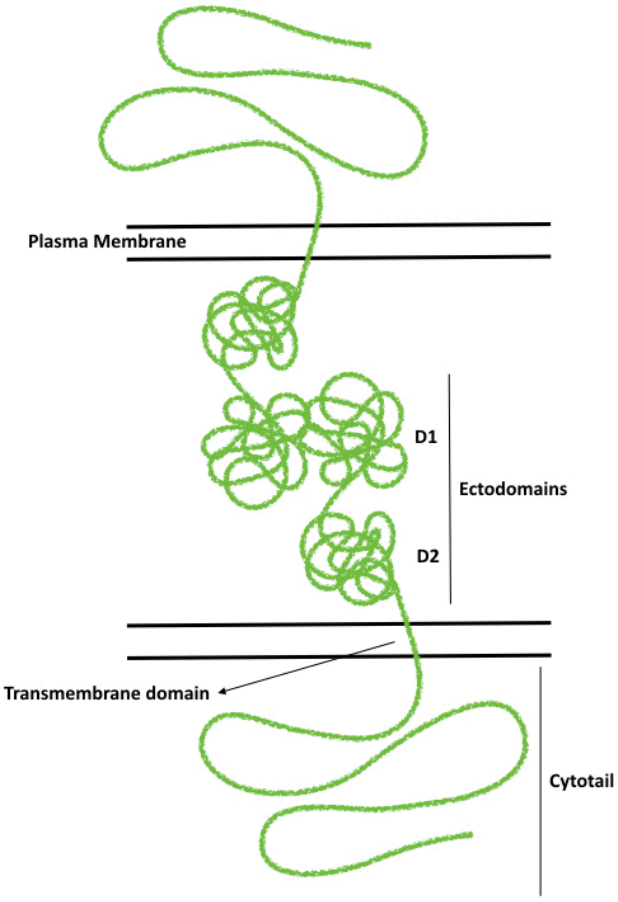


Fig.1.5. (A) **Schematic diagram of Coxsackie and Adenovirus Receptor structure.** The N-terminal comprises two immunoglobulin-like doimans, D1 and D2. A hydrophobic transmemebrane region precedes the C-terminal cytoplasmic tail. (Image Source: Carson S. D 2001)**.**

(B) **Schematic representation of CAR homodimerization *in trans* at cell:cell adhesions.** The D1 monomers undergo homophilic interactions at TJ (Raschperger et al 2006) and AJ (Hussain et al 2011).



Owing to its multifarious roles, from aiding viral infections to regulating developmental process, mediating homotypic intercellular interactions and cell adhesion formations, virologists, gene therapists, and cell biologists have all been interested in the study of CAR since its isolation by Mapoles *et al*. in 1985 (Mapoles et al 1985; Coyne, & Bergelson 2005). However, the majority of studies to date have focused on the role of CAR as a viral receptor. Studies over the past decade have revealed new roles for CAR in regulating epithelial junctions and signalling under homeostatic and diseased conditions (current known binding partners are summarised in Table 1.2) but little is still known about its interaction partners and mechanism of its biological functions.

Table 1.3. ***Summary of CAR interaction partners.*** Legend: * predicted binding partners

Partners	**Significance**
Zonula occluding, ZO-1	Tight junction formation & paracelluar permeability (Cohen et al 2001), mediation of homotypic cell aggregation & recruitment of ZO-1 to tight junctions (Cohen et al 2001)
JAM-C & JAM-L	Immune regulatory role in the activation of T-cells (Verdino and Wilson, 2011; Garrido-Urbani et al 2014)
β-catenin	Tight junction stability (Walters et al 2002)
MUPP1	Tight junction stability (Coyne et al 2004)
Alpha-catenin*	Interaction not validated but predicted to mediate cell proliferation, migration, invasion, and morphology (Stecker et al 2009)
Actin	Cytoskeletal reorganisation & cell migration (Huang et al 2007)
Tubulin	Cell migration (Fok et al 2006)
ligand-of-numb protein-X	Cell development (Sollerbrant et al 2003)
Rho Kinase (ROCK)*	Predicted to regulate actin dynamics (Saito et al 2014)

MAGI-1	Uncharacterised (Ide et al 1999; Excoffon et al 2004)
PICK1	Uncharacterised (Jaulin-Bastard et al 2001; Excoffon et al 2004)
PIST *	Uncharacterised (Neudauer et al 2001; Excoffon et al 2004)
SAP97*	Uncharacterised (Muller et al 1995; Ide et al 1999; Excoffon et al 2004)
GRIP1*	Uncharacterised (Bladt et al 2002; Excoffon et al 2004)
LIN-7*	Uncharacterised (Perego et al 1999; Excoffon et al 2004)

1.3.1. *Role of CAR as a virus receptor*

The expression of Coxsackie and Adenovirus Receptor (CAR) is a determinant of the cells susceptibility to viral infections especially by the coxsackie virus B and adenovirus serotype 2 and 5 (Bergelson et al 1997). Relevant to this study are the adenovirus serotypes that infect lung epithelial cells (Shank, 1996). They contain fibre proteins protruding from the 5-fold vertices of an icosahedral capsid, the terminal region of which called the “fibre knob” are vital in its binding to high affinity receptors such as CAR (Xia et al 1994; refer to Fig.1.4 for adenovirus lifecycle). Under normal homeostatic conditions, CAR is located at the basolateral surface of epithelial cells (Carvajal-Gonzalez et al 2012). During viral infection or virus-mediated gene transfer, the fibre knob protein of the virus is bound and internalized to the cell (Wang, & Bergelson 1999; Walters et al 2001). Studies have shown that the extracellular domain of CAR is crucial in mediating its viral docking function and that the transmembrane and intracellular regions do not play a role in mediating viral infections (Excoffon et al 2005). However, the transmembrane region and the C-terminal cytoplasmic tail determine the basolateral sorting of CAR, which becomes important in minimizing the risk of viral infections by restricting the expression from the easily accessible apical (or luminal) region of polarized

epithelial cells (Pickles et al 2000). Moreover, recent studies have also shown that the intracellular domains of CAR modulate the differential entry and trafficking of adenoviruses in fibroblast-like cells (Loustalot et al 2015).

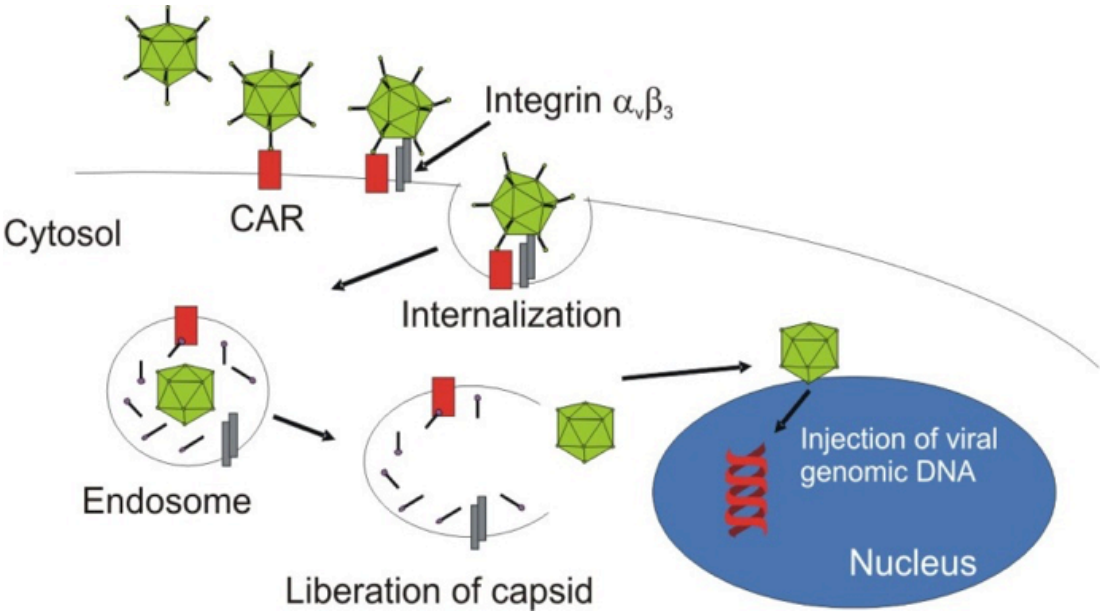


Fig.1.6. **Schematic diagram of the adenoviral life cycle.** The adenoviral fibreknob mediates interaction with CAR (Coxsackie Adenovirus Receptor). This is followed by the interaction between the viral and cell. Viral penton base then binds with the integrin αvβ3 or αvβ5 (Wickham et al 1993), and thereafter internalized, partially disassembled. Microtubules then transport the capsid to the nucleus where the viral genome is deposited. Here the expression of viral genes results in the formation of progeny, which accumulate intracellularly, subsequently causing lysis and release of progeny (Image Source: Strauss, & Strauss 2013).

1.3.2. *Role of CAR in cell:cell adhesions*

CAR has previously been shown to be present at TJ in epithelial cells, and immunoprecipitate and colocalize with ZO-1 (a TJ marker) when overexpressed in CHO cells (Cohen et al 2001). It was also observed that soluble CAR inhibited the

formation of functional TJs and virus entry into polarized epithelium also disrupted CAR dependent cell-cell junctions (Cohen et al 2001). This evidence along with CAR's adhesive functions supported a key role for CAR as an integral membrane component of TJs along with occludins, claudins and junctional adhesion molecules (JAM). Together these proteins contribute to the formation of paracellular seals that prevents movement of solutes due to their adhesive properties, thus regulating epithelial permeability and tissue homeostasis (Cohen et al 2001). Further, CAR was shown to localize underneath the TJs and co-immunoprecipitate with an AJ component, β-catenin (Walters et al 2002). Hence, the ability to form physical linkages with various adaptor proteins sub-complexes and disrupt epithelial barrier function when its homodimerisation is interrupted places CAR as a key adherens junction molecule (Cohen et al 2001; Walters et al 2002). Recent evidence from our own lab have implicated CAR as an adherens junction component, which regulates paracellular permeability and modulates adherens junction dynamics (Hussain et al 2011). We have shown that CAR colocalises with -catenin and E-cadherin in epithelial cells and PKC δ mediated phosphorylation of CAR cytoplasmic tail controls E-Cadherin localisation and recruitment at junctions (Morton et al 2013). We have also demonstrated that phosphorylated CAR is required to facilitate recruitment and transmigration of leukocytes across cell junctions during inflammation in lung epithelia (Morton et al 2016). In this case too the phosphorylation of CAR is triggered by the cytokine, TNFα via a TNFR1-PI3K-PKCδ pathway. These findings suggest an additional role for CAR in maintaining junctional homeostasis. Interestingly, CAR at AJ was also found to facilitate epithelial transmigration of leukocytes and neutrophils by acting as the docking receptor for JAM-L (Zen et al 2005; Verdino et al 2010). These studies also demonstrated that the transepithelial

resistance was unaltered at tight junctions. We have also shown that CAR may play a dual role in epithelial transmigration of leukocytes by facilitating direct adhesion and inducing signaling changes to permit efficient leukocyte passage across junctions (Morton et al 2013). These findings suggest that CAR can regulate AJ dynamics as well as acting as a responsive molecular regulator of extracellular events in homeostasis and disease.

1.3.3. *CAR in intracellular signalling*

As well as forming homodimers through interactions with itself *in trans*, CAR has also been shown to bind to a number of proteins through the C-terminal domain as discussed above and summarized in Table 1.2; these include TJ proteins PICK1, MUPP1 and ZO-1 (Cohen et al 2001; Coyne et al 2004; Excoffon et al 2004). CAR has also been proposed to bind to the actin and microtubule cytoskeletons (Huang et al 2007). As well as binding partners, the C-terminal domain has also been shown to regulate intracellular signaling cascades. Previous studies have shown a role for CAR in activation of p44/42 MAPK and JNK signaling pathways in different cell types (Cunningham et al 2003; Tamanini et al 2006; Marchant et al 2009). Work from our laboratory has also shown that CAR can control epithelial cell adhesion through activation of p44/42 MAPK (Farmer et al 2009). Increased activation of β1 and β3 integrins, that are essential for cell:matrix adhesion in epithelial cells, was found to be a direct consequence of CAR-induced p44/42 activation. It was also demonstrated that the cytoplasmic domain of CAR is required for p44/42 activation, integrin activation and CAR localisation to cell junctions (Farmer et al 2009). Moreover, data from our lab and others has shown that overexpression of CAR can disrupt the recruitment and localization of E-cadherin to epithelial cell:cell junctions

(Hussain et al 2011; Caruso et al 2010). These studies suggest that CAR can potentially act to co-ordinate the signaling between cell:cell and cell:matrix adhesion sites to maintain epithelial cell integrity.

1.3.4. *Role in cell migration*

As previously described CAR interacts directly with scaffolding proteins, ZO-1 and Multi-PDZ Domain Protein-1 (MUPP1) located at tight junctions (Cohen et al 2001). Both of these proteins bind to F-actin, therefore the interaction of CAR with these two proteins implicates CAR in the potential co-ordination of cytoskeletal dynamics. Indeed, CAR has been suggested to interact directly with F-actin (Huang et al 2007). It was also shown to contribute to F-actin bundling in neurons and suggested to associate with the F-actin regulatory molecule Rho Kinase (ROCK) (Saito et al 2014). CAR has been implicated in regulating migration in U87 glioma cells where it binds tubulin through its cytoplasmic tail (Fok et al 2007). Our lab also showed that CAR is also involved in the transepithelial migration of leukocytes under inflammatory conditions. The cytoplasmic tail of CAR is phosphorylated both *in vitro* and *in vivo* specifically in response to TNFα through a PI3K and PKCδ pathway, which facilitates the leukocyte movement (Morton et al 2016) thus playing an indirect role in facilitating migration of other cell types.

1.3.5. *Role in inflammation*

Previous studies in rat cardiomyocytes have shown that CAR expression was upregulated under inflammatory conditions (Ito et al 2000). Recently our lab demonstrated that although CAR expression levels remain unchanged, it was hyper-phosphorylated *in vivo* in both acute and chronic lung inflammation models

(Morton et al 2016). This study demonstrated that *in trans* homodimers of CAR located at cell:cell adhesions are phosphorylated in response to exogenous TNFα. This aids in the transmigration of monocytes and neutrophils, both *in vitro* and *in vivo*, suggesting that CAR may be an important receptor in the control of inflammation (Morton et al 2016). However, conflicting data documented in primary cultures of murine hippocampal neurons and adult murine neural progenitor cells incubated with TNFα and IFN-γ in a dose-dependent manner showed reduction in CAR expression levels. These data suggest a tissue specific role for CAR in inflammatory condition but nonetheless its cellular localisation and association with JAMs makes it an potentially important candidate in controlling local immune responses (Verdino et al 2011; reviewed in Ortiz-Zapater et al 2017).

1.4. **Migration mechanisms in Epithelial Cells**

Cell migration is a complex fundamental process required in a range of different stages in organisms, including tissue morphogenesis during development, wound healing and immunosurvelliance (reviewed in Vicente-Manzanares et al 2005; & Trepat et al 2012). Crucial to cell migration is the synchronisation of the cell's structural framework, the cytoskeleton along with membrane remodelling and cell: matrix adhesions. Of the key molecular players essential for cell migration, cell:matrix adhesions and F-actin are indispensable as described in section 1.2.1 above. Broadly cell migration can be divided into four steps (i) protrusion of the front or leading edge to form the lamellipodia through increased polymerisation of cytoskeletal protein, actin (Ballestrem et al 2000; Carlier et al 2003; Le Clainche, & Carlier 2008) (ii) formation focal adhesions to stabilise the lamellipodia (Webb et al 2002) (iii) repositioning of the nucleus and microtubule-organising centre to

determine the cell polarity (iv) retraction of the trailing of leading edge of the cell aided by actin depolymerisation, actomyosin contractility and focal adhesion disassembly to translocate the cell forward (Pollard, & Borisy 2003; Mseka, & Cramer 2011; Cramer 2013). Epithelial cells undergo migration as large groups of cells through a process called collective migration. These cells retain their cell:cell contacts and apicobasal polarity to migrate in a coordinated manner during processes such as gastrulation, wound healing and tumour spreading (Poujade et al 2007; Friedl et al 2009; Chuai et al 2012; Scarpa, & Mayor 2016; reviewed in Mayor, & Etienne-Manneville 2016). While the five basic steps of migration is common to both modes of cell movement, collective migration is characterised by the following distinct features and modifications:

- Only cells located at the front of the group or cluster called the “leader cells” are polarised and involved in determining the directionality and speed of cell movement. They form continuous lamellipodia that overlap the boundaries of neighbouring cells and drive the leading edge forward (Farooqui, & Fenteany 2005). The role of E-cadherin based cell:cell junctions is crucial in this mode of migration to form protrusions and sense external environment (Li et al 2012; Bazellières et al 2015). Due to the presence of cell:cell adhesive structures, the remaining cells in the group, i.e., “follower cells”, do not form classical leading edges and hence remain unpolarised with their migration being dictated by the leader cells. However, follower cells can actively influence the polarisation and directionality of leader cells (reviewed in Mayor, & Etienne-Manneville 2016). The designation of leader and follower cells is simply dependent of their relative position in a migrating group, and can be interchanged. During wound healing, leader cells are

formed in a Rho-dependent manner and Erk1/2 activity drives migration (Omelchenko et al 2003; Chapnick, & Liu 2014).

- At the wound edge of leader cells mature focal adhesions are assembled. These associate with actomyosin cables to promote the contraction of the cell body.

- Leader cells generate the traction forces necessary to drag the follower cells along in a Rho-dependent manner at wound edges (Li et al 2012; Reffay et al 2014). Cell:cell or adherens junctions between leader cells are connected to the thick actomyosin cables that are involved in transmitting mechanical forces across the cell cluster. It has been shown that follower cells to contribute to the overall traction force to drive the migration (Trepat et al 2009).

1.5. **Filamin A**

Filamin is a key F-actin crosslinking protein and is crucial to maintain the cell's stability and elasticity. Filamin plays a major role is regulating signaling by organizing actin architecture in response to changes in the extracellular matrix (ECM) and therefore, serves as a versatile molecular scaffold in cell signalling and motility. (Nakamura et al 2007).

This 160nm long semi-flexible protein comprises of two 280kD monomers. The structure of Filamin A is shown in Figure 1.7. The molecule comprises of a highly conserved N-terminal spectrin-related actin binding domains (ABD) comprising of two calponin homology sequences (CH1 and CH2) (Nakamura et al 2011). This is followed by 24 β-pleated sheet repeats (IgFLN 1-24) – a rigid chain of Ig repeats (Ig1-15) termed as 'rod1' with a low affinity for actin binding; and a compact globular 'rod2' domain. The rod2 domain consists of equivalent number of Ig repeats (Ig16-23), which pair with the neighbouring repeat (Ig 16, 18 and 20 with 17,19 and 21 respectively) (Playford et al, 2010). There is a similarity in structural composition of the 15-immunoglobulin repeats of rod1 domain, which is known to actively participate in F-actin binding. The globular compact rod2 domain does not contribute to F-actin binding or branching, but is instead involved in partnering interactions with signalling molecules, transmembrane receptors and adaptor proteins (Popowicz et al 2006; Nakamura et al 2007). The last repeat Ig24 mediates homodimerization (Nakamura et al 2007; Baldassarre et al 2009). Two hinges separate the Ig repeats 15 and 16 (hinge 1) and repeats 23 and 24 (hinge 2) (Gardel et al 2006).

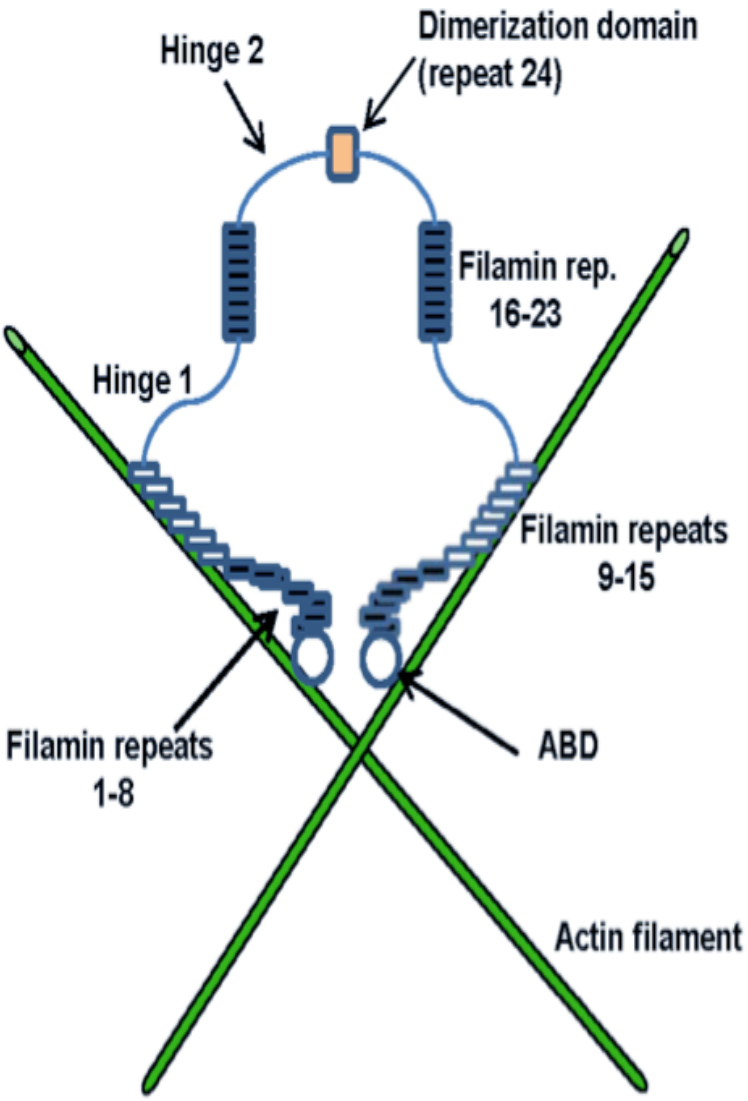


Fig.1.7. (A) **Schematic representation of FLNa structure**. The actin-binding N-terminal domain is followed by 24 immunoglobulin-like repeats of ~96 amino acids each. They are interrupted by two hinge regions (Hinge 1 and Hinge 2, which fold into antiparallel β-sheets. The IgFLN 24 repeat at the C-terminal is the dimerization domain. Repeats 1–15 make up rod domain 1, and repeats 16–24 make up rod domain 2. Domain pairs 16–17, 18–19, and 20–21 are boxed in gray. (Image Source: Yue et al 2013)

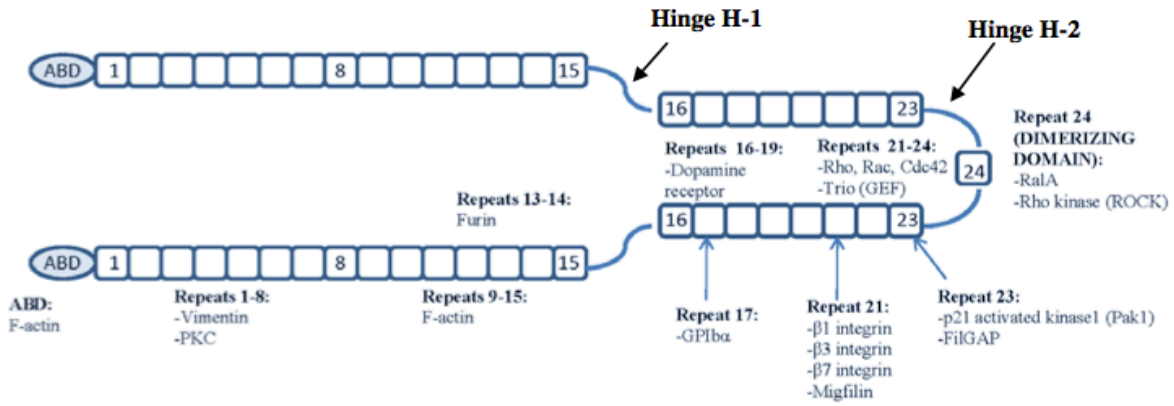


Fig.1.7.(B) **Schematic representation of the FLNa binding partners and their site of interaction.** (Image Source: Kim, & McCulloch 2011).

1.5.1. *Role of Filamin A in regulating of F-Actin*

Filamin A provides stable yet flexible structural cross-links between F-actin filaments with contractile networks, thus aiding actin assembly. It binds to F-actin through its ABD and initiates either perpendicular or high angle branching of F-actin. The distal half of rod1 (IgFLN 9-15) augments in actin binding, while the FLN 24 Ig repeats in C-terminus aid in the branching activity (Nakamura et al 2007). FLNa dimers, having different binding affinities to F-actin, diverge at specific high angles to form L-shaped structures (Nakamura et al 2007). The high affinity subunit binds F-actin, while the unbound subunit accommodates a second F-actin if it is available in the correct perpendicular orientation (Zhou et al 2010; Figure 1.7). The elasticity of hinge 1 and branching activity of the distal region of rod1 together engage F-actin in high angle branching.

Apart from regulating actin dynamics through direct binding, through its association with cell signalling molecules, Filamin A is actively involved in rearranging actin

cytoskeleton. For example, it binds and targets FilGAP, a Rac-specific GTPase binding protein, to membrane protrusions where the latter is involved in lamellipodia formation (Nakamura et al 2009). This interaction is also crucial in determining actin remodelling during cell spread (Ohta et al 2006). Filamin A 's association with p-21 activated kinase-1 is also important in membrane protrusion and ruffle formation (Vadlamundi et al 2002). Therefore, filamin acts as an integrator of cell signalling and actin dynamics (reviewed in Stossel et al 2001; Feng, & Walsh 2004; Nakamura et al 2011). It is also thought to integrate the three major cytoskeletal systems-actin, microtubule and intermediate filament networks through its close association with proteins such as vimentin (Brown, & Binder 1992; Kim, & McCulloch 2011). It is, thus, unsurprising loss of Filamin A can disrupts cell migration, cell polarization and spreading (Cunningham et al 1992; Fox et al 1998; Simpson et al 2008; Baldassarre et al 2009) thereby impairing tissue development and morphogenesis (Feng et al 2006; Hart et al 2006).

Table.1.4. ***Summary of Filamin A binding partners.*** Legend: * binding site on FLNa from N-terminal (top) to C-terminal (bottom) unless otherwise noted; ** direct interaction has not been confirmed (reviewed in Nakamura et al 2011; Yue et al 2013).

Partners	**Binding sites***	**Significance**
Cytoskeleton and cell shape maintenance		
F-actin	ABD, Rod-1	FLN induces orthogonal F-actin networks with unique mechanical and physiological (Hartwig, & Stossel 1975; Nakamura et al 2007)
Calmodulin	ABD	Regulates F-actin binding in vitro (Nakamura et al 2005)

R-Ras	3	Enhances integrin activation (Gawecka et al 2010)
Syk	5	Flna is required for ITAM-mediated receptor signaling in platelet (Falet et al 2010)
Vimentin	1–8	Expression of IgFLNa1-8 restores spreading of filamin-deficient HEK-293 cells, vimentin phosphorylation, and the cell surface expression of β1 integrins (Kim et al 2010)
Supervillin	8–10, 20–22	Overexpression of IgFLNa8–10, but not 20–22 decreases spreading of Hela cells on fibronectin (Smith et al 2010)
FILIP	15–18	Downregulates FLNa and controls polarity and migration of neocortical cell (Nagano et al 2002)
ICAM-1	19–24	Transendothelial migration (Kanters et al 2008)
Membrane and membrane associated proteins		
Dopamine D2 and D3 receptors	19	Stabilizes β-arrestins-filamin-A complex (Lin et al 2001; Kim et al 2005)
Pro-Prion	10,16–18, 20, 21, 23	FLNa interacts with the GPI anchor peptide signal sequence of pro-PrP that is expressed in some cancer cells This interaction also promotes cell spreading and migration of melanoma cells (Li et al 2009; Li et al 2010)
GPIbα (CD42b)	17	Platelet adhesion and activation Genomic instability (Fox et al 1985; Nakamura et al 2006; Li et al 2007)
Integrin β	21	Adhesion Mechanoprotection Negative regulation of integrin activation (Kiema et al 2006; Sharma et al 1995)
Tissue Factor	22–24	Phosphorylation of TF enhances the interaction (Ott et al 1998)

CEACAM1 (CD66a)	23–24	Reduces cell migration (Klaile et al 2005)
Migfilin (FBLP-1)	21	Disconnects FLNa from integrin and promotes talin-integrin binding (Tu et al 2003; Lad et al 2008; Ithychanda et al 2009)
Caveolin-1	22-24	Intracellular trafficking (Stahlhut et al 2000; Muriel et al 2011)
FAP52(PASCIN2/ Syndapin II)	15–16	Formation of focal adhesion (Nikki et al 2002)
Small GTP-binding proteins and their regulators		
Trio	23–24	GEF for RhoG/Rac1 and RhoA Required for ruffling (Bellanger et al 2000)
FilGAP	23	Rho- and ROCK-regulated GAP for Rac. FLNa-binding is required for cell spreading and stimulates GAP activity (Nakamura et al 2009; Ohta et al 2006)
Rho	24	Remodeling of cytoskeleton (Ohta et al 1999)
Rac	24	Remodeling of cytoskeleton (Ohta et al 1999)
Cdc42	24	Remodeling of cytoskeleton (Ohta et al 1999)
RalA	24	Filopodia formation (Ohta et al 1999)
ROCK	24	Remodeling of cytoskeleton (Ueda et al 2003)
Intracellular signalling		
β-arrestins	22	ERK activation and actin cytoskeleton reorganization (Scott et al 2006)
Wee1	22-24	Regulates Wee1 expression and promotes G2/M phase progression (Lian et al 2012)
Androgen receptor	16-19	Required for androgen-induced cell migration (Ozanne et al 2000; Castoria et al 2011)
TRAF2	15-19	Inflammatory signal transduction (Leonardi et al 2000)

Sphingosine kinase 1	22–24	FLNa-dependent kinase activity (Maceyka et al 2008)
Lbc**	Unknown	RhoGEF Co-immunorecipitated with calcium-sensing receptor, RhoA, and Gαq (Pi et al 2002)
P190RhoGAP**	Unknown	Expression of calpain-insensitive FLNa excludes p190RhoGAP from the lipid raft, thereby increase Rho activity (Mammoto et al 2007)
Vav-2**	Unknown	Guanine nucleotide exchange factor for the Rho family Complexes with Rac (Del Valle-Perez et al 2010)
Nuclear function associated proteins		
BRCA1	23-24	Facilitates the recruitment of BRAC1 and RAD51 to DNA damage sites and stabilizes the DNA-PK holoenzyme (Velkova et al 2010)
BRCA2	21-24	Required for efficient homologous recombination DNA repair and recovery of G2/ M phase arrest (Yuan et al 2001; Men et al 2004)
RefilinB	15-24	Stabilizes perinuclear actin actin networks and regulates nuclear shape (Gay et al 2011)
TIF-IA, RPA40	ABD	Suppresses ribosomal RNA gene transcription (Deng et al 2012)
TAF1B/mKIAA1093	1-7	Possible role in rRNA production, protein translation and the organization of centromeres (Qiu et al 2011)
p311**	Unknown	Highly expressed in invasive glioma cells and enhances glioma cell migration (McDonough et al 2005)

1.5.2. *Interactions with cell:cell adhesion proteins*

Membrane and cell junctional proteins are also known to directly or indirectly associate with Filamin A (summarised in Table 1.4). Filamin A acts as a ligand for caveolae-associated protein, calveolin-1 and is believed to be involved in the spatial organisation of caveolar membranes and actin (Stahlhut, & van Deurs 2000). Filamin A was found to directly bind calcium-sensing receptor (CaR), RhoA and Trio at cell adhesion junctions thereby acting as a scaffold to mediate CaR-Trio-E-Cadherin and E-cadherin-catenin adhesion complexes, both of which are important to maintain cell:cell adhesions (Tu et al 2008; Tian et al 2011; Tu, & You 2014).

1.5.3. *Interactions with cell:matrix adhesion proteins*

Filamin A has long been known for its interaction with β subunits of integrin through which it stabilises the inactive conformation of the receptor (Loo et al 1998; Liu et al 2015; Truong at al 2015). It interacts with a number of adaptor proteins such as talin and migfilin to regulate integrin-based adhesion assembly, integrin affinity and recycling (Kiema et al 2006; Legate, & Fässler 2009; Das et al 2011; Truong at al 2015). Filamin also coordinates the interaction between cytoskeletal component, F-actin and integrins (Popowicz et al 2006).

1.6. **Filamin inactivates β1 integrins**

Studies have shown that the loss of FLNa (and FLNb) impairs initiation of cell migration and reduces cell speed (Baldassarre et al 2009). In HT1080 cell lines, Baldasarre et al., (2009) observed that Filamin A double knockdowns (FLNabKD), although recused by re-expressing full length FLNa, could not be rescued by FLNΔ19-21 (present in the rod2 region). This suggests that the ABD region and the

IgFLN19-21 are essential for the initiation, if not the entire process of cell migration. Two β chain cytoplasmic tails of clustered integrins bind to the IgFLN21 repeats on the FLNa dimers (Pfaff et al 1998; Liu et al 2015 ; Nakamura et al 2007). Further, Gehler et al. found that FLNa-β1 binding complex functions as a link between the ECM and cytoskeletal actin, mechanically and biochemically (Gehler et al 2009). This coupling of FLNa and integrin is also known to regulate cell morphogenesis in response to signals from and composition of ECM. It was then observed that integrin mediated migration was reduced by its tight binding to FLNa (Calderwood et al 2001). Hence, FLNa- β1 integrin complex influence migration. Calderwood et al. (2001) proposed that this complex controls the formation of transient membrane protrusions and cell polarization. However, migfilin, a Filamin A binding protein found in cell-cell and cell-matrix contact sites, displaces Filamin A from β1 integrin (Das et al 2011). Thus, migfilin acts as a molecular switch that re-activates β1 integrin by disconnecting filamin (Ithychanda et al 2009).

1.7. **Aims**

1.7.1 *Hypothesis*

Our previous data has shown a role for CAR in regulating integrin signalling at cell-matrix adhesions as well as controlling cell-cell adhesion dynamics. However, the way in which CAR regulates integrin activation, and potential CAR-binding proteins that mediate this process remain unknown. We hypothesise that CAR may interact with filamin A, an integrin-inhibiting protein, through the CAR cytoplasmic tail and thus act to suppress integrin activation and regulate focal adhesion formation and homeostasis in human epithelial cells.

1.7.2. *Project Aims*

The overall aims of this study are:

- To characterize, validate and map binding of the CAR cytoplasmic tail to Filamin A.
- To analyse the potential role of phosphorylation of the CAR cytoplasmic domain in regulating Filamin A binding.
- To determine the role of CAR in controlling epithelial cell focal adhesion assembly.
- To establish whether CAR acts as a molecular switch in regulating cell:cell and cell:matrix adhesions.

2. *Materials &*

Methods

2.1. Materials

Table 2.1.1. ***Cell lines used for Cell culture and Transfections.***

Mammalian Cell Lines	Source
Immortalized Human Bronchial Epithelial Cells (HBEC)	A kind gift from Dr Jerry Shay, UT Southwestern (Ramirez et al 2004)
Human Bronchial Epithelial Cells (16HBE14o$^-$)	Forbes at al 2003
Human Embryonic Kidney Cells (HEK 293FT)	A kind gift from Dr Matthias Krause, King's College London

Table 2.1.2. ***Reagents for Cell Culture and Transfection***

Reagent	Source
Serum-Free Keratinocyte Medium (Keratinocyte-SFM) with prequalified human recombinant Epidermal Growth Factor 1-53 (EGF 1-53) and Bovine Pituitary Extract (BPE)	Gibco
High Glucose Dulbecco's Modified Eagle's Media (DMEM)	Sigma-Aldrich
Minimum Essential Medium (MEM)	Sigma-Aldrich
Dulbecco's Phosphate Buffered Saline (modified, without calcium chloride & magnesium chloride)	Sigma-Aldrich
Penicillin/ Streptomycin	Gibco
L-Glutamine	Gibco
Fetal Bovine Serum (FBS)	Sera Laboratoties International Ltd.
Trypsin/EDTA	Invitrogen
Bovine Collagen, Type I	Corning®
Calcium Nitrate	Analytical Scientific
DMSO	Sigma-Aldrich
OptiMEM	Gibco
Lipofectamine 2000	Invitrogen

Effectene	Qiagen
HEPES Buffer	Gibco
DharmaFECT	GE Dharmacon
Polybrene	Sigma-Aldrich

Table 2.1.3. ***Reagents for Molecular Biology (Cloning, Plasmid Purification & GST fusion protein synthesis)***

Reagent	Source
One Shot® TOP10 Chemically Competent *E.coli*	Invitrogen
BL21(DE3) Competent Cells	Aligent Technologies
Luria Bertani (LB) agar/broth	Sigma-Aldrich
Ampicillin sodium salt	Sigma-Aldrich
Kanamycin sulfate	Sigma-Aldrich
Zeocin™	Invitrogen
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich
Protease inhibitor cocktail	Calbiochem
Brij-35	Thermo Scientific
L-Glutathione reduced	Sigma-Aldrich
Glutathione Sepharose™ 4 Fast Flow Beads	GE Healthcare
QIAGEN® Plasmid *Plus* Midi Kit	Qiagen
QIAGEN® Plasmid *Plus* Mini Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
UltraPure™ Agarose	Invitrogen
SafeView	NBS Biologicals

Table 2.1.4. ***Reagents for pulldown assay and immunoprecipitation***

Reagent	Source
GFP Trap Beads	ChromoTek
A/G-Agarose Beads	Santa Cruz Biotachnology, Inc.
DNase-I	NEB
NuPAGE™ 4-12% Bis-Tris Protein Gels	Thermo Scientific
Pierce™ Silver Stain	Thermo Scientific

Table 2.1.5. ***Reagents for Immunofluorescence***

Reagent	Source
Phosphate Buffered Saline (PBS)	PAA
Immersol 510 Immersion oil	Zeiss
FluorSave reagent	Calbiochem

Table 2.1.6. ***Reagents for Western blotting***

Reagent	Source
Pierce* BCA* Protein Assay Kit	Thermo Fisher Scientific
Calyculin A (phosphatase inhibitor)	Sigma-Aldrich
β-mercaptoethanol	Sigma-Aldrich
Tween-20	Sigma-Aldrich
Acrylamide/Bis-acrylamide, 30% solution	Sigma-Aldrich
Sodium Dodecyl Sulfate (SDS), 10% solution	Thermo Fisher Scientific
N,N,N’,N’-Tetramethylethylenediamine BioReagent (TEMED)	Sigma-Aldrich
Novex™ Empty Gel Cassettes, 1.5mm	Invitrogen
Amersham Protran Supported 0.2µm NC	GE Healthcare

Bovine Serum Albumin (BSA)	Sigma-Aldrich
Nonfat-Dried Milk Bovine	Sigma-Aldrich
Clarity™ Western ECL Substrate	Bio-Rad

Table 2.1.7. ***Primary antibodies used for Western blot (WB), Immunoprecipitation (IP) and Immunofluorescence (IF)***

Primary Antibody	Dilution (Use)	Host Species	Source
Anti- Filamin1	1:1000 (WB) 1:400 (IF)	Mouse	Santa Cruz Biotechnology, Inc.
Anti- GAPDH	1:2000 (WB)	Mouse	Santa Cruz Biotechnology, Inc
Anit-HSC 70	1:2000 (WB)	Mouse	Santa Cruz Biotechnology, Inc
Anti- Vinculin	1:1000 (WB) 1:400 (IF)	Mouse	Sigma-Aldrich
Anti-GFP	1:400(IF); 1µL/sample (IP)	Rabbit	MBL
Anti-GFP	1:2000 (WB)	Mouse	Roche
Anti- CAR (H300)	1:1000 (WB); 1:100 (IF)	Rabbit	Santa Cruz Biotechnology, Inc.
Anti- p-thr290/ser293CAR polyclonal antibody	1:200 (WB); 1:50 (IF)	Rabbit	In-house – produced by Perbioscience (Thermofisher)
Anti-E-Cadherin [HECD-1]	1:200 (IF)	Mouse	Abcam
Anti- Total β1 integrin β1 (Clone CD29)	1:1000 (WB); 1:400 (IF)	Rabbit	Epitomics
Anti- Active β1 integrin (Clone 12G10)	1:400 (IF)	Mouse	Epitomics
Anti- Active β1 integrin (Clone 9EG7)	1:400 (IF)	Mouse	BD Pharmingen™
Anti-Flag	1:2000 (WB); 1:400 (IF)	Rabbit	Sigma-Aldrich

Table 2.1.8. ***Secondary antibodies and fluorescent dyes used for Western blot (WB) and Immunofluorescence (IF)***

Conjugated antibodies	Dilution (Use)	Species	Source
Anti-Rabbit HRP	1:1000(WB)	Goat	DAKO
Anti-mouse HRP	1:1000(WB)	Goat	DAKO
Alexa Fluor® 488 goat anti-mouse IgG	1:400(IF)	Goat	Invitrogen
Alexa Fluor® 568 goat anti-mouse IgG	1:400(IF)	Goat	Invitrogen
Alexa Fluor® 647 goat anti-mouse IgG	1:100(IF)	Goat	Invitrogen
Alexa Fluor® 488 goat anti-rabbit IgG	1:400(IF)	Goat	Invitrogen
Alexa Fluor® 568 goat anti-rabbit IgG	1:400(IF)	Goat	Invitrogen
Alexa Fluor® 568 goat anti-rat IgG	1:500(IF)	Goat	Invitrogen

Fluorescent Dyes	Dilution (Use)	Source
Alexa Fluor® 488 phalloidin	1:250(IF)	Invitrogen
Alexa Fluor® 568 phalloidin	1:250(IF)	Invitrogen
Alexa Fluor® 647 phalloidin	1:250(IF)	Invitrogen
Hoechst 33342 (10 mg/mL)	1:4000 (IF)	Invitrogen

Table 2.1.9. ***Reagents for fabrication of elastic substrates***

Reagent	Source
SYLGARD® 184 Silicone Elastomer Kit	Dow Corning
SYLGARD® 527 A&B Silicone Dielectric Gel	Dow Corning
Ethanol, Absolute	Thermo Fisher Scientific

Table 2.1.10. ***Buffers and solutions used for Western blotting, Immunoprecipitation and GST pull down assays. All reagents used were from Sigma unless otherwise stated.***

Buffers/ Solutions	Composition
15% SDS-PAGE gel	15% 30%-acrylamide mix, 400 mM Tris pH 8.8, 0.1% SDS, 0.1% ammonium persulphate (APS), 0.05% TEMED
10% SDS-PAGE gel	10% 30%-acrylamide mix, 400 mM Tris pH 8.8, 0.1% SDS, 0.1% ammonium persulphate (APS), 0.05% TEMED
8% SDS-PAGE gel	8% 30%-acrylamide mix, 400 mM Tris pH 8.8, 0.1% SDS, 0.1% ammonium persulphate (APS), 0.05% TEMED
Coomassie brilliant blue R250 solution	0.025% coomassie brilliant blue R250, 40%methanol, 7% acetic acid, adjust volume to 1L with dH_2O
Coomassie destaining solution	50% Methanol, 10% Acetic acid, 40% water and 0.05 g Brilliant Blue
GST-Protein Wash Buffer	Dissolve 11.6 g NaCl, 1.95 g Tris-Cl and 0.5 ml Brij-35 in 500 mL 1xPBS and adjust pH to 7.4
IP Buffer	50 mM Tris Base, 150 mM NaCl, 1 mM EDTA, 50 mM Sodium fluoride, 1% NP-40, 1% (for pull down assay) / 0.2% (for immunoprecipitation) Triton X-100, adjust pH to 7.4 and volume to 1 L with dH_2O
RIPA Buffer	10 mM Tris Base, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, adjusted to pH 7.4 and volume to 1L
2% SDS Buffer	50 mM Tris-Cl, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 2% SDS, adjust pH to 7.4 and volume to 1 L with dH_2O
PBS-T (1x)	10 tablets of Phosphate buffered saline, 0.5% Tween 20

SDS Sample Loading Buffer (2X)	60 mM Tris-HCl (pH 6.8), 25% Glycerol, 2.5% Sodium dodecyl sulphate (SDS), 0.02% Bromophenol blue
SDS Sample Loading Buffer (5X)	150 mM Tris-HCl (pH 6.8), 62.5% Glycerol, 6.25% Sodium dodecyl sulphate (SDS), 0.05% Bromophenol blue
TBS-T (10x)	20 mM Tris-base pH 7.5, 150 mM NaCl, 0.1% Tween 20
Running Buffer (10x)	Dissolve 30 g Tris Base (Thermo Fisher Scientific), 144 g Glycine and 10 g SDS in 1 L dH_2O
Transfer Buffer (10x)	Dissolve 30 g Tris Base (Thermo Fisher Scientific) and 144 g Glycine in 1 L dH_2O

2.2 Methods

2.2.1. *Cell Culture*

Immortalized Human Bronchial Epithelial Cells (HBEC) were grown in Keratinocyte Serum-Free Medium (KSFM) supplemented with 5% (w/v) pituitary extracts, 0.0005% (w/v) epidermal growth factor (rEGF; both supplements provided by the manufacturer) and 1% antibiotics (penicillin/streptomycin). 16HBE cells were grown in MEM containing 10% FBS and 1% antibiotics (penicillin/streptomycin). HEK 293FT cells were cultured in DMEM containing 10% FBS, 1% L-glutamine and 1% antibiotics (penicillin/streptomycin). All cells were maintained at 37°C in a humidified incubator with an atmosphere containing 5% CO_2. Stocks of all cells lines were retained in liquid nitrogen in media with 10% DMSO and 20% FBS.

2.2.2. *Passaging of cells*

(a) *Human Bronchial Epithelial Cells (HBEC)*

Adherent HBEC were cultured until ~75% confluent, typically in a 25 cm^2 tissue culture coated with 0.1 mg/mL solution of bovine collagen type I diluted in 0.02 N acetic acid. The growth medium was removed and cells washed with 5 ml PBS (without Mg^{2+} and Ca^{2+}) before being incubated with in trypsin/EDTA at 37°C until they detach. 5 ml of KSFM was added to the cells and centrifuged at 1200 rpm for 3 minutes at room temperature. 1:3 dilution of the cells resuspended in 6 ml of KSFM was placed in fresh tissue culture flask coated with collagen (followed by PBS washes) and volume made up to 5 ml with KSFM. All media was warmed to 37°C and collagen coated flasks washed with PBS (without Mg2+ and Ca2+) before use.

(b) *16HBE cells*

16HBEs were cultured until ~75% confluent, typically in a 75 cm^2 tissue culture coated with 10% FBS. The growth medium was removed and cells washed with 10 ml PBS (without Mg^{2+} and Ca^{2+}) before being incubated with in trypsin/EDTA at 37°C until they detach. Cells were resuspended with growth media and replated at 1:3 dilution in fresh tissue culture flask coated with 10% FBS and volume made up to 15 ml with MEM. All media was warmed to 37°C before use.

(c) *HEK 293FT cells*

The same protocol as described in (b) was used for passaging HEK 293FT cells; however, they were grown on plastic and replated at 1:10 dilution.

2.2.3. *Cryopreservation of Cell Lines*

Cells cultured to until ~75% confluency were washed with PBS (without Mg^{2+} and Ca^{2+}), resuspended in the appropriate growth media and centrifuged at 1200 rpm for 3 minutes. The cell pellet was collected and resuspended in 700 μl of growth media. 200 μl DMSO and 100 μl FBS were added and the cell suspension was thoroughly mixed and transferred onto to ice. Cells were frozen at -80°C for up to a week and then transferred into liquid nitrogen tanks.

2.2.4. *Plasmid purification*

E.coli cells transformed with the appropriate plasmid were incubated at 37°C with shaking in 150 ml of antibiotic containing Luria Bertani (LB) broth overnight. Cells were harvested by centrifugation at 4000 rpm for 10 minutes at 4°C. Plasmids were then purified using high-yield protocol of QIAGEN® Plasmid *Plus* Midi Kit according to

the manufacturer's instructions. Purified DNA samples were pelleted, air-dried and resuspended in water. The concentrations were determined using a Nanodrop ND-1000 Spectrophotometer.

2.2.5. *GST Fusion Protein Synthesis*

E.coli cells were transformed with GST-tagged cytoplasmic tail (CT) of CAR and CT mutants of CAR (summarized in Table 3.2.1; refer to Fig.3.2 for schematic diagram of CAR highlighting unique protein motifs/ amino acid residues on its cytotai) DNA constructs. The bacterial cultures were cultured in LB media supplemented with 100 μg/ml ampicillin at 37°C. Overnight cultures were diluted in 1:50 into 500 ml of LB broth supplemented with ampicillin and grown at 30°C with shaking for about 2 hours, until the optical density reached 0.6-0.8. The bacterial cultures were then induced for 4 hours with 1 mM IPTG. Bacteria were pelleted at 4000 rpm for 15 minutes before being resuspended in 10 ml of PBS with 1% protease inhibitor cocktail. The cells were disrupted by probe sonication following which BRIJ at 1% concentration was added and mixed well. They were then centrifuged at 4000 rpm for 15 minutes to remove cell debris. The supernatant was then incubated with glutathione sepharose beads (pre-washed with PBS) for 4 hours or overnight. The beads were collected by centrifugation at 4000 rpm for 5 minutes, washed twice with PBS containing 0.4 M NaCl (Sigma) and 1% BRIJ (Sigma), resuspended in 1 ml PBS and stored at 4°C.

Table 2.2.1. ***CAR Cytoplasmic tail mutants used in this study***

CAR Mutant type	Abbreviation	Mutation Site
Non-phosphorylated mutants		
Double mutant	AA	T290A & S293A
Single mutants	T>A/ S>A	T290A/ S293A
Phospho-mimetic mutants		
Double mutant	DD	T290D & S293D
Single mutants	T>D/ S>D	T290D/ S293D

2.2.6. *Cloning and Site-directed mutagenesis*

(a) *CAR plasmids*

Full length CAR was cloned in frame into pHR9SIN-SEW lentiviral expression vector, which was a gift from Dr Adrian Thrasher (Institute of Child Health, UCL) and into pGEX-2T to generate GST-fusion proteins by Dr. PE Morton as described in Morton et al, 2013. Phospho-mutant constructs were generated using site directed mutagenesis.

(b) *ABD-Flag transient expression plasmid*

Full length Actin Binding Domain (ABD)-GFP (a kind gift from Dr. Calderwood, Yale University) was amplified by PCR using the GGC AAA GCG GCC GCA TGA GTA GCT CCC ACT CTC GGG CG (forward) and TAT AGC GGC CGC CCC AAG GGA GCC CCT GGC TTC A (reverse) primers to generate a 5' NotI and a 3' NotI restriction site (underlined). The PCR product was then cloned into c-Flag pCDNA3.1 (Addgene) to generate a C-terminal Flag tag.

All constructs were verified by sequencing prior to use.

2.2.7. *DNA transfection*

(a) *Using Lipofectamine 2000*

Human Embryonic Kidney 293T cells were used for transfection of filamin constructs. Cells were plated at 50% confluency in Corning® tissue-culture treated 60mm petri-dishes. 2 μg of DNA was diluted in 500μl of OptiMEM (solution A) and 5 μl of Lipofectamine 2000 was diluted in 500 μl of OptiMEM (solution B). Solutions were incubated for 5 minutes at room temperature. Solution B was gently mixed into solution A (hereafter, referred to as transfection mixture) and incubated at room temperature for 30 minutes. Cells were washed with 4 ml OptiMEM after removing the growth media. Tranfection mixture was added drop wise to the cell plate and shaken gently. Cells were incubated at 37°C for 5 hours, after 1.5 ml of OptiMEM was added to the plate. Transfection medium was replaced with complete growth media and re-incubated at 37°C for 12-24 hours before plasmid expression was observed.

(b) *Using Effectene*

HBEC and 16HBE monolayers were transfected with transient expression plasmids using Effectene. Cells were plated at 60-70% confluency in Corning® tissue-culture treated 60 mm petri-dishes. 1 μg of DNA was diluted in Buffer EC along with 16 μl of Enhancer, vortexed for 1 second and incubated for 5 minutes at room temperature. 24 μl of Effectene Transfection Reagent was added to the mix, vortexed for 10 seconds and incubated for 10 minutes at room temperature. The transfection mixture was then added to cells containing fresh complete media and incubated at 37°C for 24-48 hours before plasmid expression was observed.

2.2.8. *Silencing CAR expression*

(a) CAR knockdown using retroviral short hairpin (sh) RNA

Hairpin sequence:

TGCTGTTGACAGTGAGCGCGCGCTTTGAATATGAAATCATTAGTGAAGCCACAGATGTAATGA

TTTCATATTCAAAGCGCTTGCCTACTGCCTCGGA

100 nM of the shRNA was transfected into HEK 293FT cells using lipofectamine 2000 as described in 2.2.7(a). After 24 hours, OptiMEM from HEK cells were collected and replaced with growth media. The OptiMEM collected was centrifuged at 1200 rpm for 5 minutes to pellet any cells/ debris. The supernatant was used to infect 16HBE cells seeded at 50% confluency along with polybrene at 8 μg/ml. This procedure was repeated thrice over 48 hours. 16HBE cells were then selected for the plasmid in growth media containing 2 μg/ml puromycin.

(b) CAR knockdown using siRNA

Commercially available CAR knowdown siRNA (CAR siRNA (m): sc-39919 from Santa Cruz Biotechnology, Inc.) was diluted to 5 μM working stock. 5 μl of 5 µM siRNA and 2.5 μl of DharmaFECT reagent were diluted in 95 μl and 97.5 μl of pre-warmed OptiMEM and incubated at room temperature for 5 minutes. The two solutions were then mixed and incubated for a further 20 minutes at room temperature. Growth media on 16HBEs plated at a confluence of ~50% in a 6-well plate were washed and replaced with 1300 μl OptiMEM. The transfection mixture was added to these cells and incubated at 37°C in 5% CO_2. After 6 hours, cells were replaced with growth media and incubated for 48 hours before checking for CAR expression by western blotting.

2.2.9. *Western Blotting*

Cells were cultured until 80% confluent in 25cm^2 tissue culture flasks and lysed in 1 mL of RIPA buffer/ 2% SDS buffer containing protease inhibitor cocktail (1:100) and Calyculin A (1:1000). Lysates were incubated for 10 minutes on ice after thoroughly mixing them. Cell lysate proteins were collected as supernatant after centrifuging at 10,000 rpm for 10 minutes at 4°C. An equal volume of SDS sample loading buffer (2X) containing β-mercaptoethanol (1:50) was added to cell lysate proteins and boiled at 95°C for 5 minutes and centrifuged to clear any remaining debris. The lysates were used immediately or stored at -20°C. 40 μl of total cell lysate proteins were loaded in each well on 8% SDS-PAGE gels and subjected to SDS-PAGE. Proteins were blotted onto nitrocellulose membranes using Invitrogen XCell II™ transfer apparatus. Blots were then blocked in 5% milk/PBS-T (or 5%BSA/TBS-T for phospho antibodies) one hour at room temperature and incubated with primary antibodies overnight at 4°C or for 2 hours at room temperature. They were washed three times for 10 minutes with PBS-T/TBS-T prior to incubation with horseradish peroxidase-conjugated secondary antibodies for one hour at room temperature. They were then washed a further three times and the immunoprobed proteins were visualized using ECL kit, on Bio-Rad GelDoc XR imaging system. For re-probing, blots were treated with Re-blot Plus for 10 minutes, blocked for 30 minutes and incubated overnight with primary antibodies at 4°C.

2.2.10. *GST-pulldown assays*

Cells were cultured until 80% confluent and lysed in 1 mL IP buffer (inclusive of 2% NP-40 and 2% Triton X-100) containing protease inhibitor cocktail (1:100) and Calyculin A (1:1000). Lysates were incubated for 10 minutes on ice after thoroughly

mixing them. Cell lysate proteins were collected as supernatant after centrifuging at 10,000 rpm for 10 minutes at 4°C. 50 μl of each lysate was kept aside for use in loading controls while the rest were incubated with pre-washed GST beads overnight at 4°C. Following incubation the unbound fractions were removed and the beads washed three times in IP buffer before being boiled for 5 minutes in 50 μl of 2X SDS sample loading buffer containing β-mercaptoethanol (1:50). 40 μl of samples were loaded onto 8% polyacrylamide gels and immunoblotted using anti-Filamin A antibody or anti-GFP antibody.

2.2.11. *Mass spectrometric Analysis*

GST pulldown assay (described in 2.2.10) of HBEC using purified GST-tagged CAR cytotail mutants (refer to 2.2.5) was employed to precipitate binding partners of CAR. Pulldown lysates were resolved on NuPAGE™ 4-12% Bis-Tris Protein Gels and silver stained using Pierce™ Silver Stain for Mass Spectrometry according to the manufacturer's instructions. Resolved protein bands of interested were cut and gel slices were sent to Aberdeen Proteomics Facility, University of Aberdeen, where Liquid Chromatography–Mass Spectrometry analysis was employed to identified potential protein hits (refer to Fig. 3.1).

2.2.12. *Immunoprecipitation*

Method A:

Cells were harvested at 90% confluence in 1 ml lysis buffer containing protease inhibitor cocktail (1:100), Calyculin A (1:1000) and DNase I enzyme (1:1000). Lysates were thoroughly pipetted and incubated on ice for 10 min prior to incubated for 3 h with 5 μl of prewashed GFP-Trap beads per sample.

Method B:

Cells were harvested 90% confluence in 1 ml lysis buffer containing protease inhibitor cocktail (1:100) and Calyculin A (1:1000). Lysates were incubated on ice for 10 min prior to centrifugation at 10,000 g for 10 min. Supernatant was collected and incubated overnight with 40 μl of prewashed A/G beads coupled to anti-GFP (from MBL) or IgG (control; Dako) antibody per sample.

50 μl of lysate/supernatant was retained for analysis of cell lysate. 10 μl 5x loading buffer containing β-mercaptoethanol (1:50) was added. Beads were washed four times and boiled in 80 μl 2x loading buffer containing β-mercaptoethanol (1:50). 40 μl samples were separated on SDS-PAGE gels, transferred onto nitrocellulose membrane, blocked in 5% milk in PBS-T and probed with the indicated primary antibodies followed by detection with horseradish peroxidase (HRP)-conjugated secondary antibodies. Blots were developed with an ECL kit and chemiluminescent signal detected and quantified using a Bio-Rad XR system and ImageLab software.

2.2.13. *Treatment with F-actin depolymerizing agents*

HBEC or 16HBE plated on collagen or 10% FBS coated coverslips at ~90% confluency were washed and provided fresh complete media. Cytochalasin D was added at a final concentration of 25 nM (Wakatsuki et al 2001) and Latrunculin B at a final concentration of 2.3 μM, and incubated for 45 minutes at 37°C before fixing with 3.6% formaldehyde solution. DMSO of appropriate concentration was used as control. Cells were then immunostained (as described in section 2.2.16) and imaged on a Nikon Eclipse T*i* A1R confocal microscope. Images acquired were scored for CAR-FLNa colocalisation as a single-blind experiment.

2.2.14. *Adhesion Assay*

HBEC were seeded at 1x10^3 cells in Corning® 12-well plate coated with collagen. Cells were allowed to adhere for 45 minutes before fixing with 3.6% formaldehyde and staining with Hoechst 33342. Fluorescent images were acquired on Evos FL Auto 2 flourescent microscope (Invitrogen). 9 × 9 tile-scans were obtained using a 10 × air objective using identical camera acquisition times. Tiles were knit into .TIFF files using FIJI software and total cell count was obtained by thresholding for nuclear stain followed by automated counting.

2.2.15. *Scratch Wound Healing Assay*

HBEC monolayers grown in Corning® 6-well plate coated with collagen were gently scratched with a P10 tip to introduce a wound. Cells were then washed with PBS (without Mg^{2+} and Ca^{2+}) thrice before adding fresh growth media inclusive of 25 mM HEPES. Time-lapse experiment was set up using Olympus IX71 Inverted Flourescence & Phase Contrast Microscope using a 10x objective. Images were acquired at intervals of 10 minutes over a 12-hour time period. The resulting movies were analysed in FIJI to determine the starting 'wound' area and quantified as percentage of wound closure over time.

2.2.16. *Time-lapse assay to quantify lifespan of cell junctions*

HBEC were seeded at ~30% confluency in Corning® 6-well plate coated with collagen and incubated at 37°C overnight. Cells were replaced with fresh growth media inclusive of 25 mM HEPES and calcium at 1.25 mM. Time-lapse experiments were set up using Olympus IX71 Inverted Flourescence and Phase Contrast Microscope. Images were acquired using a 10x objective at intervals of 10 minutes over a 12-

hour time period. Cells were then manually tracked in FIJI to determine the duration for which two cells formed and maintained a physical contact, i.e, cell junction as shown in Fig. 4.11.

2.2.17. *Fabrication of elastic substrates*

Polydimethylsiloxane (PDMS) from Dow Corning, Sylgard 527 gel and Sylgard 184 elastomer, were blended to create substrates with tunable mechanical properties based on the mass ratios described in Palcheesko et al, 2012. As per manufacturer's directions, Sylgard 527 was prepared by mixing equal weights of part A and part B using a vortex for 5 minutes at maximum speed followed by 2 minutes of defoaming at 1000 rpm. This 1:1 mass ratio of Sylgard 527 component yielded 5 kPa gels. Sylgard 184 was prepared per manufacturer's directions by mixing 10 parts base to 1 part curing agent using the same mixing and defoaming cycle. For 50 kPa gels, pure Sylgard 527 and 184 were prepared as described above, and then combining Sylgard 184:527 by mass ratio of 1:10 followed by an additional mixing and defoaming cycle. Once mixed, the PDMS was either poured into 60 mm diameter petri dishes to create, ~1 mm thick films for Co-IPs or spin-coated onto 16x16 mm glass coverslips at 1,000 rpm to create, ~1mm thick films using POLOS Spin Coater. All dishes and coverslips were cleaned with 70% ethanol and using PDC-32G-2 Plasma Cleaner (Harrick Plasma) by prior to coating them with PDMS gels. All PDMS was cured at 60°C overnight (12-18 hours) for all experiments. PDMS coated coverslips were coated with collagen for 1 h at R.T. or overnight at 4°C.

2.2.18. *Preparation of samples for Confocal Imaging*

HBEC were plated onto collagen-coated 13mm coverslips 4-well plates. 16HBE cells and HEK 293FT/CHO-1 cells were plated on coverslips coated with 10% FBS and directly on glass, respectively. Calcium (in the form of calcium nitrate) was added at a final concentration of 1.25 mM where needed. Media was removed 48 hours after seeding, and coverslips washed once with PBS before fixation in 4% paraformaldehyde in PBS for 15minutes. After washing thrice with PBS, cells were permeabilized using 0.1% Triton X-100 in PBS for 10 minutes. Coverslips were incubated with filtered primary antibody for 2 hours at room temperature. They were washed twice with PBS-Tween and once with PBS before incubating with appropriate fluorescent-conjugated secondary antibody. After extensive washing once in each PBS-tween, PBS and water, coverslips were mounted on glass slides with FluorSave to prevent bleaching and allowed to air-dry overnight.

2.2.19. *Confocal Imaging*

Images of cells were acquired on confocal microscope (Nikon Eclipse T*i* A1R inverted). Emission wavelengths of 405 nm, 488 nm, 561 nm and 640 nm from Nikon's LUN laser units were used. Images were captured using CFI Plan Apochromat Lambda Series Objectives- 40x, 60x oil immersion and 100x oil immersion (Nikon). Images were exported from database file .nd2 to TIFF files and processed with FIJI software.

2.2.20. *Image Processing:*

(a) *Quantifying number and size of focal adhesions*

Images acquired on the confocal microscope were used to count particles of size 0.2-3.0 μm^2 by an in-built plugin after setting a constant threshold value on FIJI. The summarised results generated by the software containing information about focal adhesion count, total area and average size were imported into Microsoft Excel. The number of cells present in each image divided the focal adhesion count to generate values that were used to plot bar graphs. Results were expressed as the mean ± SEM. from the specified number of experiments indicated in the figure legends. Student T-test was applied to analyse statistical significance and P value presented in the figure legend.

(b) *Quantifying cell velocity and persistence*

Cell speed and persistence were calculated using the tracking data of individual cells obtained from experiment described in section *2.2.14*. Cell tracks were analysed using Chemotaxis Tool Plugin in FIJI.

2.2.21. *Statistical analysis*

Results are expressed as the mean ± SEM from the specified number of independent experiments, as indicated in the figure legends. Student's t-test or ANOVA were used to analyse statistical significance as appropriate and detailed in the figure legends.

3. CAR Binds to Filamin A in Human Epithelial Cells

3.1. **Introduction**

Coxsackie and Adenovirus receptor (CAR), a member of the JAM family of adhesion receptors, is located at both tight and adherens junctions between epithelial cells, where it assembles adhesive contacts through homodimerisation in trans (Cohen et al 2001; Farmer et al 2009). Expression levels of CAR are known to affect cell-cell adhesion, migration and cancer progression (Walters et al 2002; Fok et al 2007; Anders et al 2009; Reeh et al 2013). However, the recruitment, binding partners and signaling effects of CAR at cell junctions and the mechanisms by which CAR acts to co-ordinate cell:cell and cell:matrix adhesions remain poorly understood.

Previous work from our lab have showed CAR regulates paracellular permeability and modulates adherens junction dynamics (Hussain et al 2011). We have shown that CAR colocalises with beta-catenin and E-cadherin in epithelial cells and PKC δ mediated phosphorylation of CAR cytoplasmic tail controls E-Cadherin localisation at junctions (Morton et al 2013). These findings suggest an additional role for CAR in maintaining junctional homeostasis. It was also shown that the viral docking receptor, CAR, localizes to AJ and regulates E- Cadherin dynamics (Hussain et al 2011). Together these AJ molecules work towards mediating effective communication between cells and maintain cell polarity.

As previous described in section 1.8, Filamin A (FLNa) is not only required for organising actin architecture but also serves as a versatile molecular scaffold in cell signalling and motility (Nakamura et al 2007). It has been implicated to play a crucial role in maintaining cell junctions in vascular and cardio morphogenesis and regulating tight junction proteins at the blood-testis barrier (Feng et al 2006; Su et al 2012). It is also known to bind cell:cell adhesion proteins ICAM-1 and CEACAM1 to

regulate cell migration (Kanters et al 2008; Klaile et al 2005). FLNa plays a crucial role in the formation of calcium-sensing receptor, CaR-Rho A-Trio complex that recruits E-cadherin to maintain cell:cell adhesions in keratinocytes (Tu, & You 2014).

The aims of this chapter were:

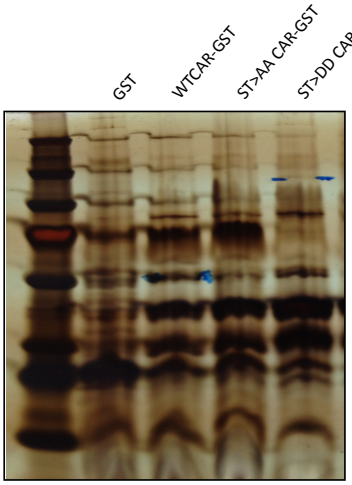
- To validate and characterise Filamin A (FLNa) as a binding partner of CAR
- To map the binding site of CAR on FLNa
- To determine the stability of the CAR-FLNa complex
- To examine the effect of external mechanical cues on CAR-FLNa binding
- To examine the importance of CAR:CAR homodimerisation *in trans* in binding FLNa

3.3. **Results**

3.2.1. ***Mass spectrometry analysis reveals F-actin crosslinker, Filamin A, as a CAR binding partner***

Previous studies have shown the cytoplasmic domain of CAR to associate with a number of junction proteins MUPP-1, ZO-1 and PICK1 (Cohen et al 2001; Coyne et al 2004; Excoffon et al 2004). Our previous data have suggested a regulatory link between junctional CAR and E-cadherin in MCF7 human carcinoma cells (Farmer et al 2009; Hussain et al 2011). However, the role of CAR in binding or influencing cell cytoskeleton has not yet been examined. Pulldown assay of confluent monolayers of WT HBEC using GST tagged CAR and its single and double phospho mutants were performed. Mass spectrometric analysis of selected protein bands from the GST pulldown assays revealed that filamin A (FLNa), an F-actin cross-linking protein, binds to the cytoplasmic tail of CAR (Fig. 3.1A & B). As shown in Fig.3.1D, FLNa is a multi-domain protein that has specific binding sites for its partners (Feng, & Walsh 2004; Nakamura et al 2011). Interestingly, CAR-binding peptide sequences identified by mass spectrometry are located in the Actin Binding Domain of FLNa, which has a high-affinity for F-Actin binding (Nakamura et al 2011; Fig.3.1C&D).

(A)



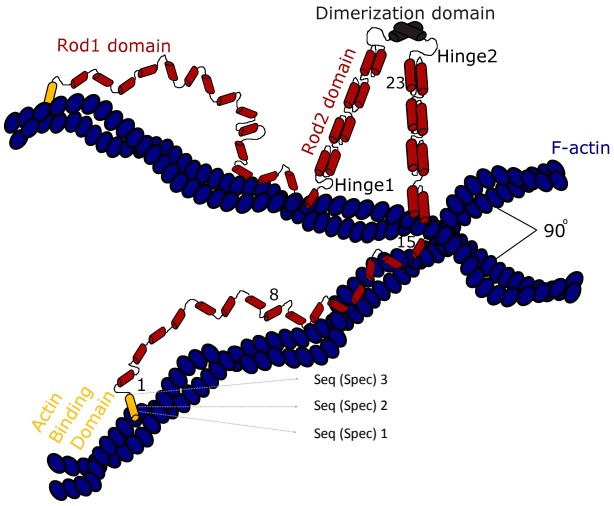
(B)

Mol. Weight	Hits
~55kDa	TCPG (Uniprot T49368) Tetraspanin 10 (Uniprot Q9H1Z9)
120kDa	MRCKgamma (Uniprot Q6DT37) PLEKHG5 (Uniprot O94827)
~270kDa	**Filamin A (uniprot P21333)** **E3 ubiquitin-protein ligase HECTD1 (Q9ULT8)**
~85kDa	Nad kinase (Uniprot O95544)

(C)

	Amino Acid Sequence
Seq (Spec) 1	WCNEHLKCVSKRI
Seq (Spec) 2	RKHNQRPTFRQ
Seq (Spec) 3	PQLPITNFSRDWQ

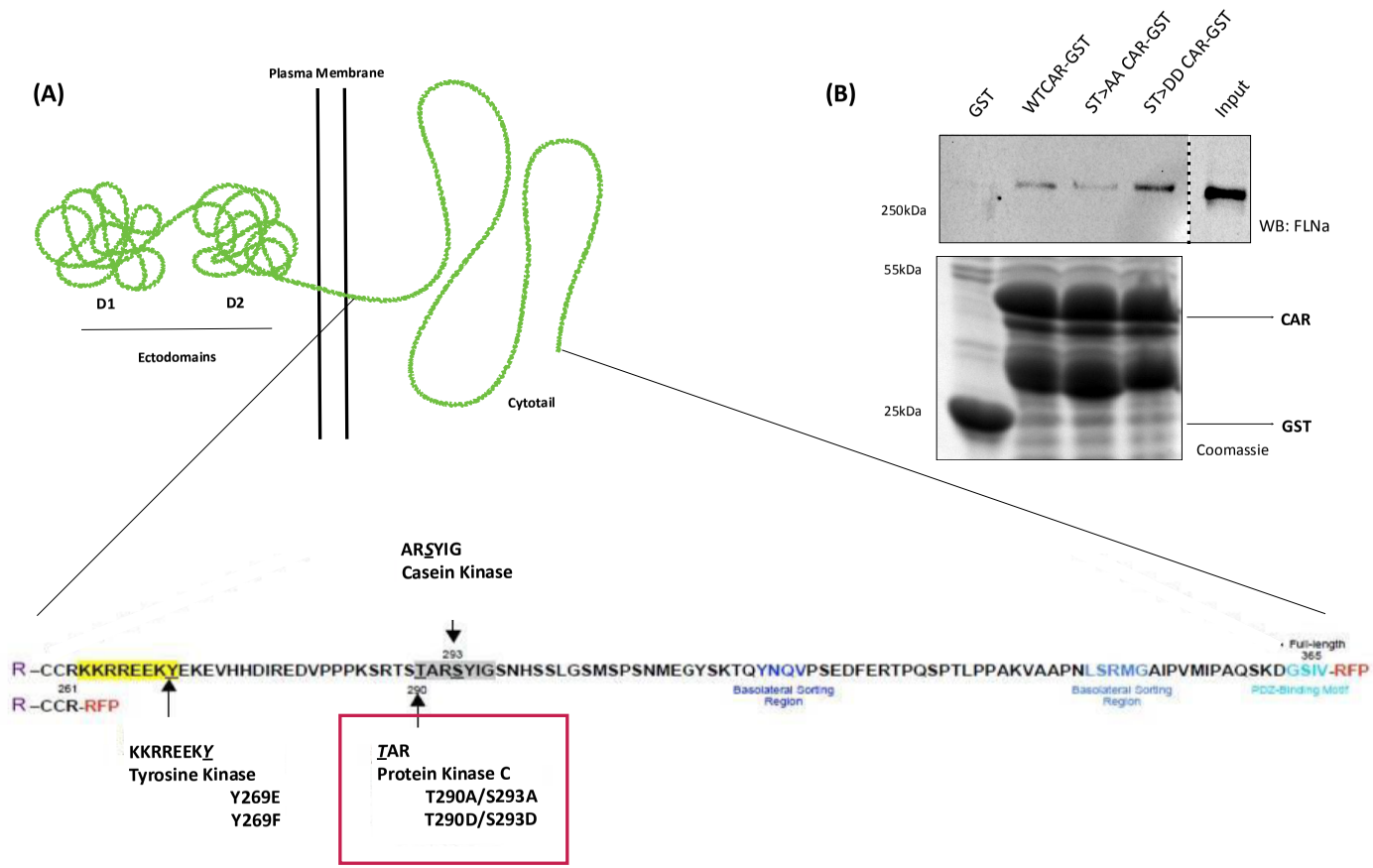
(D)



3.1. **Mass spectrometry analysis reveals FLNa as a CAR binding partner.** (A) GST-pulldown assay to investigate the interaction between CAR and Filamin A by using recombinant GST-CAR and endogenously expressed FLNa. Recombinant GST alone was used as control. Resulting samples silver stained. (B) Protein hits obtained through mass spectrometric analysis of protein bands obtained from the GST-pulldown assay. (C) Table listing the amino acid sequences obtained through mass spectrometric analysis for FLNa, one of the protein hits identified from the GST pulldown assay. (D) Schematic diagram of dimeric FLNa protein showing its various sub-domains; N-terminus Actin Binding Domain (ABD), followed by 24 immunoglobulin-like repeats categorised as Rod1 (Ig repeats 1-15), Rod2 (Ig repeats 16-23) and dimerisation unit (Ig24) at the C-terminus. Arrows indicate the position of the amino acid sequences identified in Table (C).

3.2.2. ***FLNa binding to CAR depends on CAR phosphorylation status***

Dynamic changes in the phosphorylated states of proteins regulate numerous cellular processes and signalling pathways. Three predicted phosphorylation sites were previously identified within the cytoplasmic tail of CAR; Thr290 (a predicted PKC phosphorylation motif), Ser293 and Tyr269 (Fig.3.2A). GST- tagged bacterial expression plasmids of wild-type CAR cytotail (WTCAR) and Thr290/Ser293 phosphomutant cytotails (ST>AACAR - non-phosphorylatable mutant; ST>DDCAR - phosphomimic mutant) were generated. To examine the role of CAR and its binding partner in epithelial cell integrity, immortalised human bronchial epithelial cells (HBECs) that expressed undetectably low levels of CAR were used as a model system (Ramirez et al.,2004). The ability of endogenous FLNa from wild-type (WT) HBECs to bind to the cytoplasmic tail of CAR was then tested in GST pulldown assays using lysates from WT HBEC. FLNa showed binding to WTCAR cytoplasmic tail and interestingly there was an increase in its interaction with ST>DDCAR mutant, suggesting that phosphorylation of CAR promotes FLNa binding (Fig.3.2B).



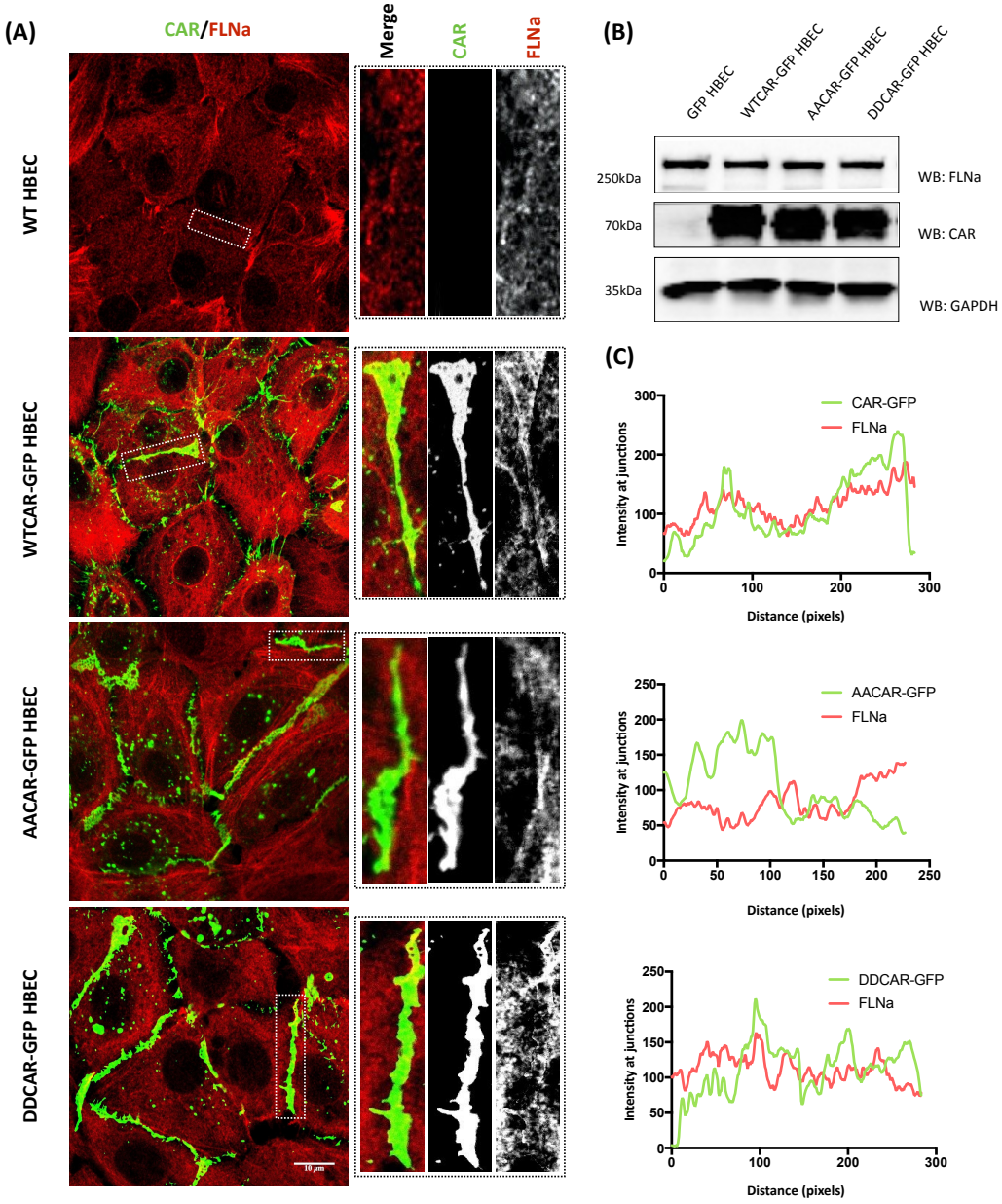
3.2. **FLNa binding to CAR depends on CAR phosphorylation status.** (A) Schematic diagram of Coxsackie and Adenovirus Receptor (CAR) highlighting unique protein motifs/ binding sites on its cytotail. (B) GST-pulldown assay to investigate the interaction between CAR and Filamin A by using recombinant GST-CAR and endogenously expressed FLNa. Resulting samples were analysed by western blotting to detect FLNa. Recombinant GST alone was used as a control.

3.2.3. ***CAR regulates sub-cellular distribution of FLNa in HBEC***

To investigate the role of FLNa-CAR interaction in epithelial cells, HBECs stably expressing WTCAR-GFP or T290/S293 phospho-mimic or non-phosphorylatable versions of CAR-GFP (DDCAR-GFP or AACAR-GFP respectively) were generated while WT HBECs were used as controls (Fig.3.3A). Western blot analysis of lysates from HBECs revealed similar global expression levels of FLNa across WT HBEC and CAR mutant cells (Fig.3.3B).

From the localisation of FLNa with respect to CAR was next analysed in WTCAR, AACAR and DDCAR-GFP expressing HBECs and control HBECs grown in high calcium containing media, which promoted the growth of epithelial monolayers with stable junctions. These cells were then immunostained for FLNa and images were acquired on a confocal microscope. FLNa in WT HEBCs was dispersed across the cell with short cables at the periphery (Fig 3A, top panels). In the WTCAR HBEC monolayers, FLNa was found to terminate and colocalise at punctate CAR in cell:cell junctions (Fig.3.3A,C) and formed longer bundles along cell periphery (Fig.3.3A). Extensions of FLNa that appeared between control WT HBECs were absent at CAR positive junctions (as shown in white box of WT HBEC images in 3.3A). In AACAR HBEC, thick bundles of FLNa were seen close to the cell periphery but not at CAR expressing junctions (Fig.3.3A,C). Conversely, increased FLNa localised at DDCAR expressing junctions (Fig.3.3A&C). The WTCAR and DDCAR-GFP HBECs, in addition to having higher levels of FLNa-CAR colocalisation at junctions, failed to form FLNa- enriched extentions between cells. These data together suggest a possible role of phosphorylated CAR in retaining filamin A at the cell periphery.

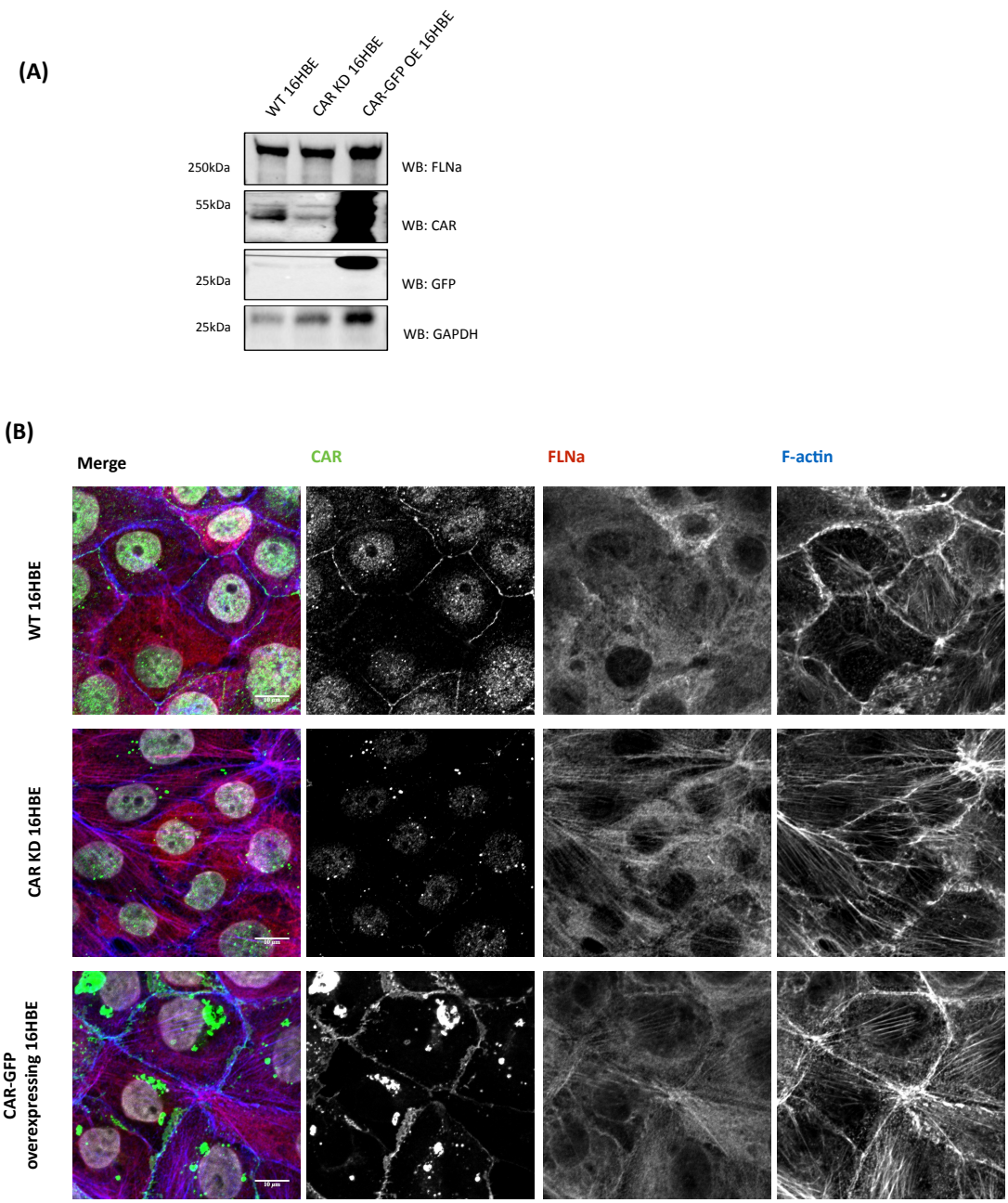
This data not only suggests that FLNa distribution was altered but that FLNa binds WTCAR and DDCAR at junctions. This association may prevent FLNa from binding to itself on neighbouring cells (due loss of intercellular FLNa extensions at CAR junctions) or other proteins.



3.3. **CAR regulates subcellular distribution of FLNa in HBEC.** (A) Confocal images of monolayers of HBECs over-expressing GFP-tagged FL-CAR and its cyto-tail mutants grown in calcium-containing media and immunostained for FLNa (grey-scale images). Side panels show WTCAR-GFP, AACAR-GFP or DDCAR-GFP expressing junctions with FLNa immunostained in red. Insets show colocalisation of CAR-GFP and FLNa at cell:cell junctions. Scale bar : 10µm. (B) Western blot of lysates from wilt-type (control), stable CARGFP and and its phospho-mutants overexpressing HBECs probed for FLNa, H300(CAR) or GAPDH as loading control Cells were lysed with 2% SDS buffer inclusive of protease inhibitors and Calyculin A. (C) Line intensity scans of FLNa (red) and WTCAR-GFP, AACAR-GFP or DDCAR-GFP at cell:cell junctions as shown in the insets in (A).

3.2.4. ***CAR regulates subcellular distribution of FLNa in 16HBE***

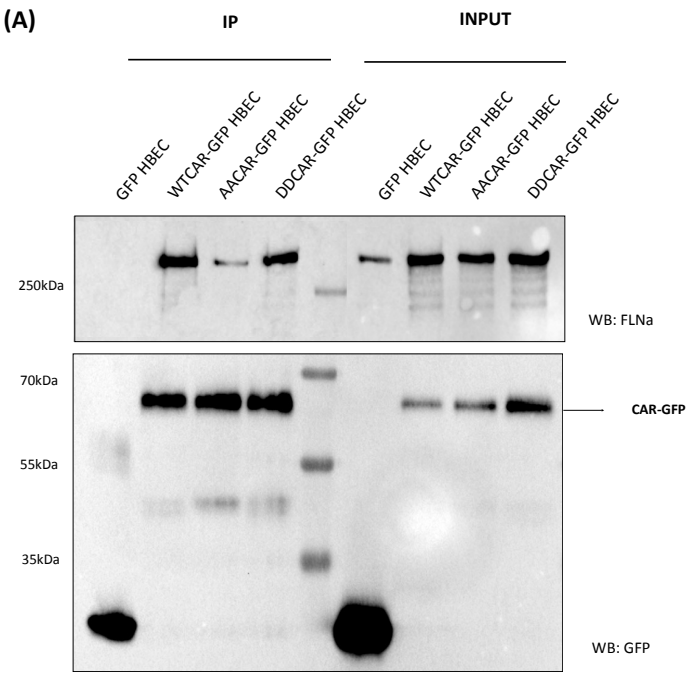
Another model system used in this study was human bronchial epithelial cell line 16HBE14o- (hereafter referred to as WT 16HBE cells; Forbes et al 2003), which expresses high levels of CAR endogenously. 16HBE lines where CAR was depleted by shRNA (CAR KD 16HBE) or over-expressing CAR-GFP (CAR-GFP OE 16HBE) were generated. Western blot analysis of lysates revealed similar levels of FLNa in all three cell lines (Fig.3.4A). Wild-type 16HBE formed monolayers with short FLNa and F-actin cables arranged haphazardly within the cell and cortical bundles around periphery (Fig. 3.4B). Knocking down CAR drastically altered spatial distribution of FLNa and F- actin that assembled into longer fibres that stretched across the cells. In CAR-GFP OE lines, increased cortical F-actin and peripheral actin belts were observed. CAR-FLNa colocalisation was seen at CAR expressing junctions as shown in Fig.3.4B. This data confirms that FLNa colocalises with CAR at cell junctions in another human lung epithelial cell line and that levels of CAR expression can alter spatial distribution of FLNa.

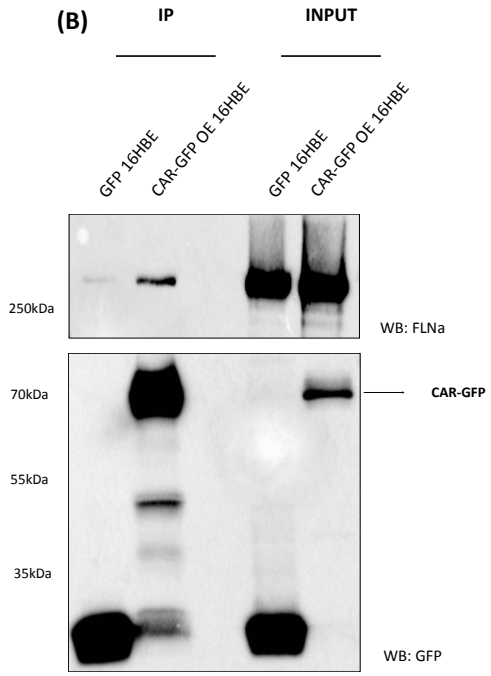


3.4. **CAR regulates subcellular distribution of FLNa in 16HBE.** (A) Western blot of lysates from wilt-type (control), CAR knockdown and stable CAR-GFP overexpressing 16HBE cells probed for FLNa, H300(CAR), GFP or GAPDH as loading control. Cells were lysed with 2% SDS buffer inclusive of protease inhibitors and Calyculin A. (B) Confocal images of monolayers of wilt-type (control), CAR knockdown and stable CAR-GFP overexpressing 16HBE cells immunostained stained for FLNa (red) and F-actin (blue). Scale bar : 10µm.

3.2.5. ***Phosphorylated CAR forms a complex with FLNa in epithelial cells***

To further examine the ability of FLNa-CAR to interact, WT, AA or DD CAR-GFP was immunoprecipitated from HBECs using GFP trap. The immunoprecipitants were analysed by western blotting and probed for FLNa to reveal high levels of binding in WTCAR and DDCAR-GFP HEBCs (Fig.3.5A). Similarly, CAR- GFP over-expressing 16HBE cells were also found to form a complex with FLNa (Fig.3.5B). Thus, it was found that FLNa and CAR are part of the same complex and that this partnership requires the phosphorylation of CAR at Thr290 and/ or Ser293 residues located in its cytoplasmic tail.

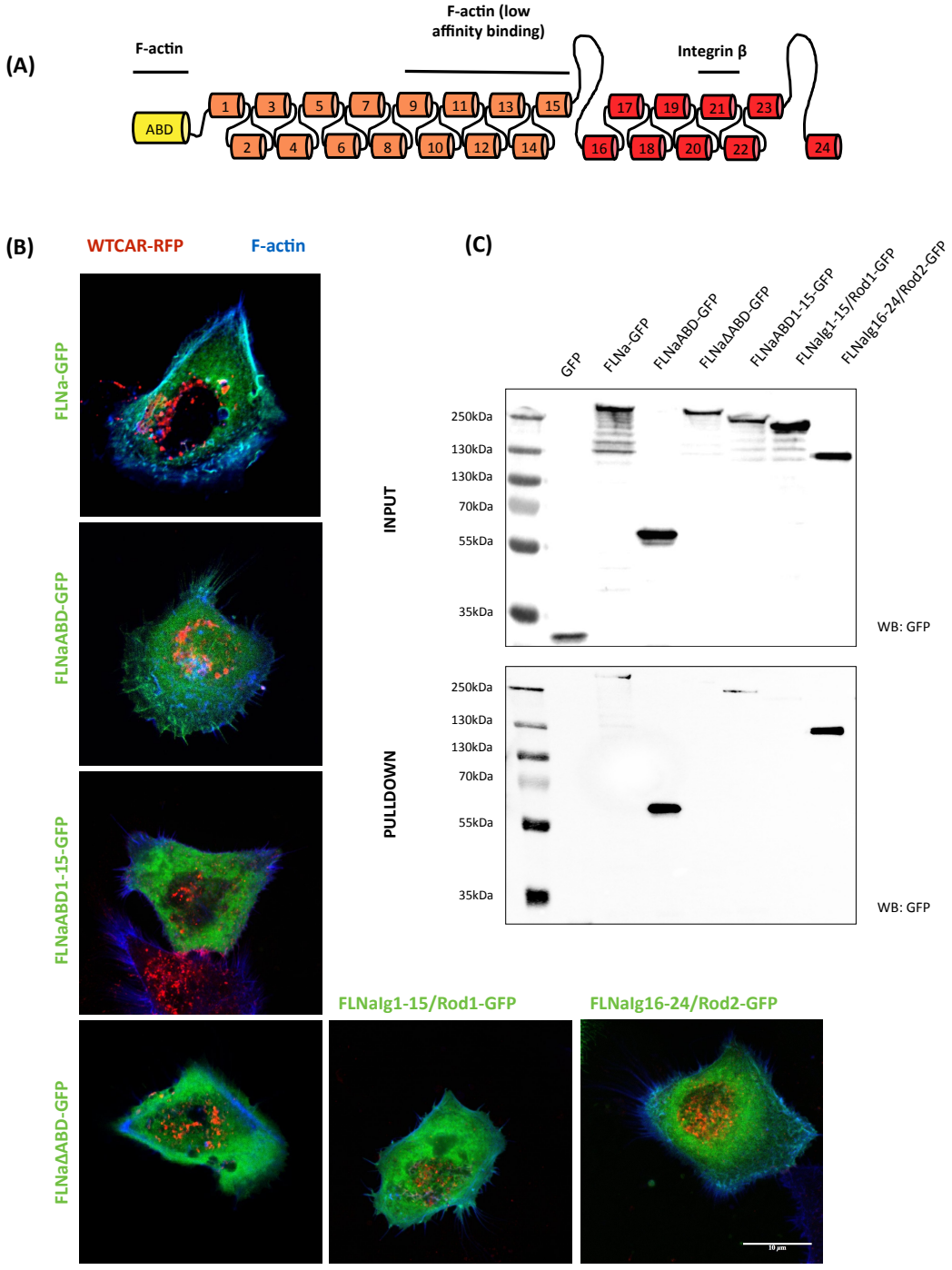




3.5. **Phosphorylated CAR forms a complex with FLNa in epithelial cells.** Immunoprecipitaion of endogenous FLNa from WTCAR-GFP, AACAR-GFP or DDCAR-GFP HBECs in (A) and CAR-GFP overexpressing 16HBE in (B), using GFP trap. Immunoprecipitates were separated by SDS-PAGE and analysed by immunoblotting for FLNa and GFP. GFP alone HBECs were used as control.

3.2.6. ***CAR associates with FLNa ABD***

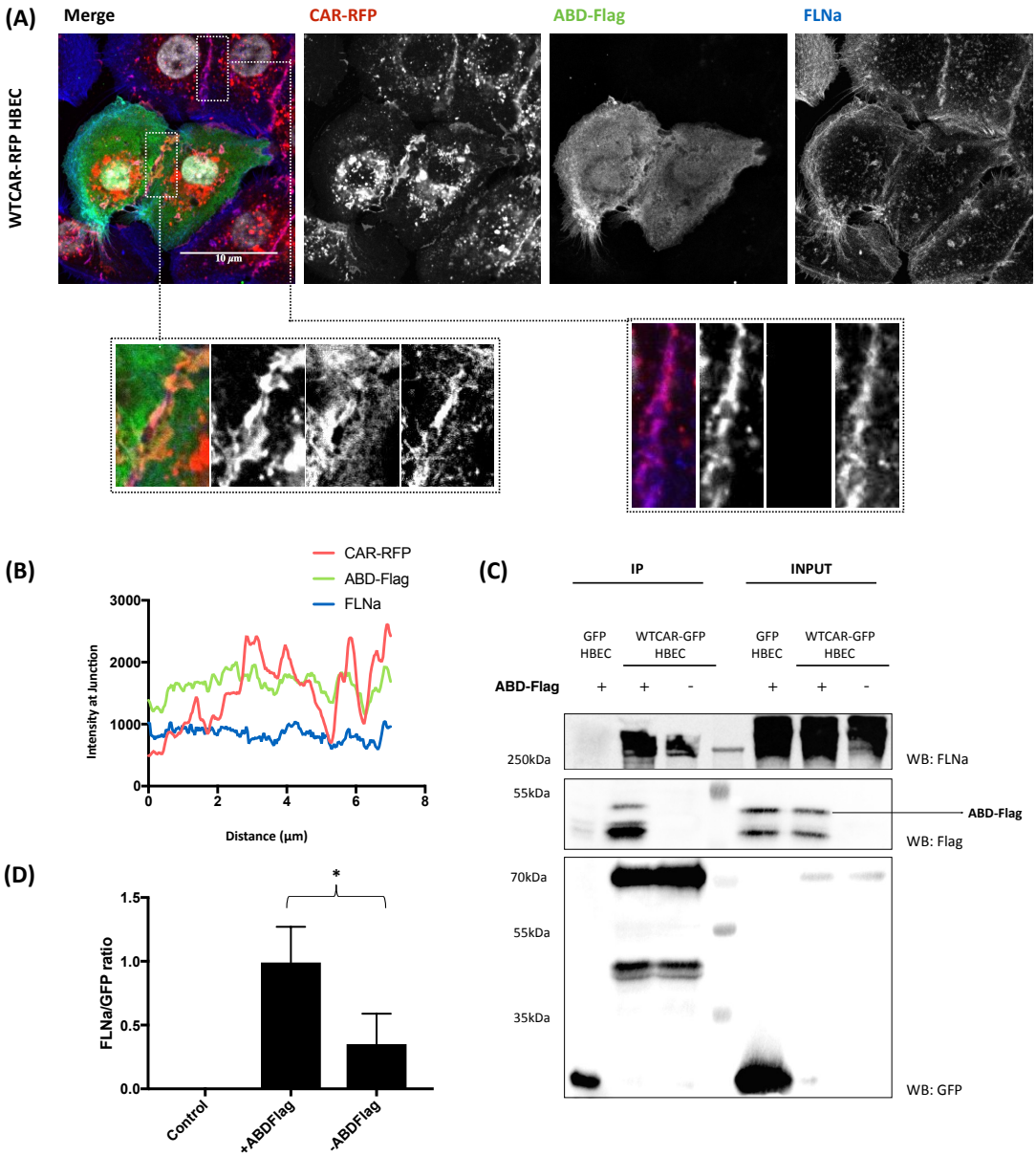
Mass spectrometric analysis revealed that with the peptides identified binding to Car were located within the Actin Binding Domain (ABD) that has a high-affinity for F-Actin binding (Fig 3.1D; Nakamura et al 2011). To better determine the interaction site between CAR and Filamin A, different domains of FLNa proteins tagged with GFP (kind gift from Dr. D. Calderwood, Yale University) were expressed in HBEC/HEK 293T cells. Transfected HBEC were immunostained for F-actin to reveal spatial distribution of FLNa and F-actin. Transfected HEK cells were analysed by GST pulldown assay with WTCAR-GST protein. Full-length FLNa and FLNa-ABD-GFP were found to colocalise with F-actin rich structures; other truncated proteins, particularly FLNaΔABD- GFP and Rod2-GFP, which do not bind F-actin (Fig.3.6A) were found diffusely localised across the cells (Fig. 3.6B). The GST pulldown assay showed high affinity of CAR cytotail to bind ABD and Rod2 domain of FLNa (Fig. 3.6C). CAR was also found to weakly bind full length FLNa and FLNa-ABD1-15 region. Together, this data indicates that although CAR has multiple binding sites on FLNa, its most readily binds the Actin Binding Domain suggesting a higher affinity of of CAR to interact with this domain.



3.6 **CAR associates with FLNa ABD.** (A) Schematic diagram of FLNa protein showing the binding sites for F-actin and integrins. (B) Confocal images of FLCAR-RFP overexpressing HBEC transiently transfected with GFP-tagged sub-domains of the FLNa protein. Cells were fixed after 24h of transfection and stained for F-actin (blue). Scale bar : 10µm. (C) GST-pulldown assay to map the binding site of CAR on FLNa by using recombinant GST-CAR to pulldown transiently expressed GFP-tagged cDNA of FLNa sub-domains in HEK 293 cells with IP buffer inclusive of protease inhibitor and Calyculin A. Resulting samples were analysed by western blotting to detect GFP expression. Cells expressing GFP alone were used as a control.

3.2.7. ***CAR colocalises with FLNa ABD and ABD overexpression reinforces endogenous CAR-FLNa complex in human epithelial cells***

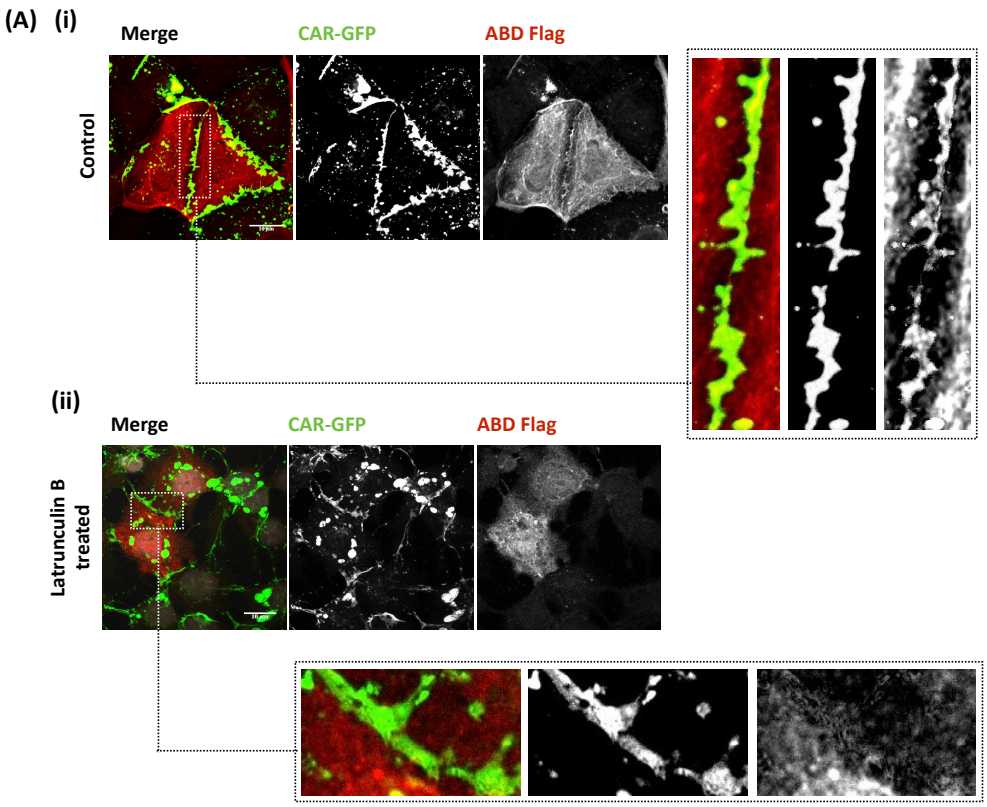
To confirm CAR-ABD interaction at cell:cell junctions, WTCAR- RFP HBECs transfected with ABD-Flag and immunostained for Flag were analysed for CAR-ABD colocalisation. ABD-Flag was found to overlap with CAR at cell: cell junctions along with FLNa as shown in Fig.3.7A,B. CAR-GFP was also immunoprecipitated from untransfected and ABD-Flag transfected WTCAR-GFP HBECs. There was twice as much FLN detected binding to CAR in the presence of ABD-Flag (Fig.3.7C,D) suggesting that ABD overexpression reinforces endogenous CAR-FLNa complex formation.

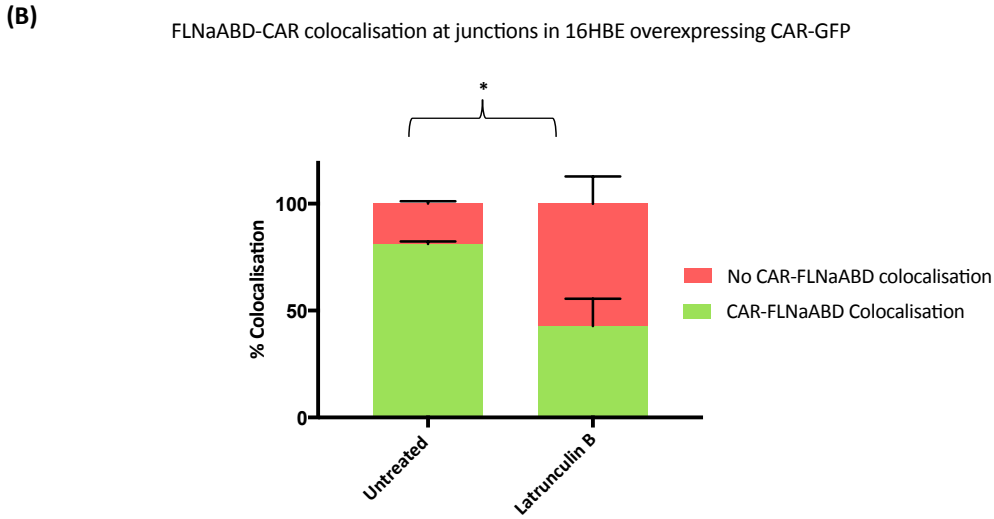


3.7 **CAR colocalises with FLNa ABD and ABD overexpression reinforces endogenous CAR-FLNa complex in human epithelial cells.** (A) Confocal images of monolayers of WTCAR-RFP HBEC transiently transfected with ABD-Flag and immunostained stained for Flag (green) and FLNa (blue). Insets show colocalisation of CAR-RFP, FLNa and/or ABD-Flag at cell:cell junctions. Scale bar : 10μm. (B) Line intensity scan of CAR-RFP, ABD-Flag (green) and FLNa (blue) at cell:cell junctions. (C) Immunoprecipitaion of endogenous FLNa from WTCAR-GFP HBECs with or without transient expression of ABD-Flag using GFP trap. Immunoprecipitates were separated by SDS-PAGE and analysed by immunoblotting for FLNa, Flag and GFP. GFP alone HBECs were used as control. (D) Quantified band intensities of chemiluminescence blots from (C) of FLNa and GFP. FLNa was normalised against immunoprecipitated GFP. N=2, data are represented as mean±s.e.m. *= p<0.05, **= p<0.005, ***= p<0.0005.

3.2.8. **Disruption of F-actin reduces the CAR-ABD interaction**

The actin binding domain of FLNa binds to F-actin with very high affinity, thereby contributing to its localisation in cells (Fig. 3.6A(ii)). We hypothesised that disruption of F-actin would result in the loss of CAR-ABD colocalisation at cell:cell junctions. Upon treatment of WTCAR-GFP HBECs transfected and immunostained for ABD-Flag with F-actin depolymerising agent, Latrunculin B, loss of some ABD-Flag was observed. Quantification of cell junctions for loss of CAR-ABD colocalisation, surprisingly, revealed that 42.5% of the cell junctions retained colocalisation of CAR and ABD (Fig.3.8B) indicating that F-actin it is not required to maintain the CAR- ABD complex at cell-cell adhesions.

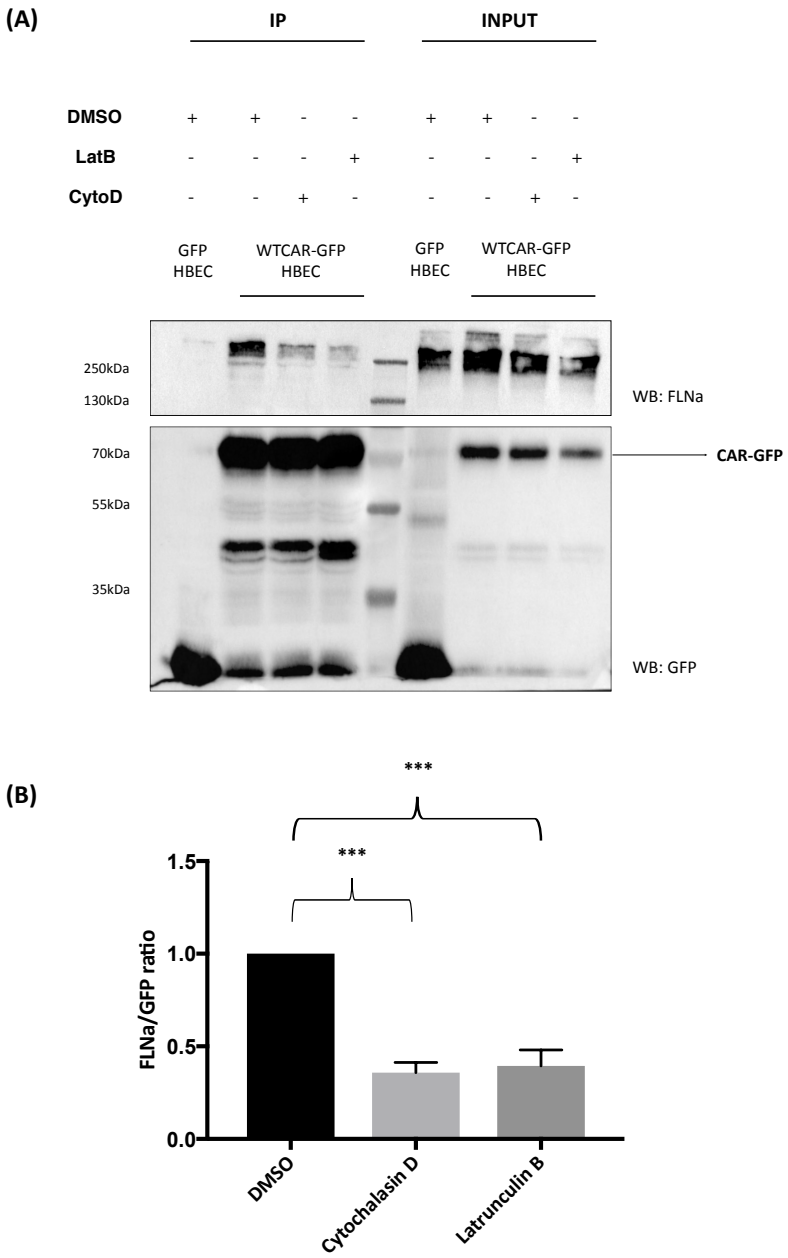




3.8 **Disruption of F-actin reduces CAR-ABD interaction** (A) Confocal images of monolayers of (i) control or (ii) Latrunculin B treated WTCAR-GFP 16HBE transiently transfected with ABD-Flag and immunostained stained for Flag (red). Insets show colocalisation of CAR-GFP and ABD-Flag at cell:cell junctions. Scale bar : 10µm. (B) Histogram of percentage colocalisation between CAR-ABD at cell:cell junctions. N=2 independent experiemnts, n>10 cell:cell junction per condition, data are represented as mean±s.e.m. Two-way ANOVA, *= p<0.05, **= p<0.005, ***= p<0.0005.

3.2.9. ***CAR-FLNa complex is reduced in cells treated with actin depolymerising agents***

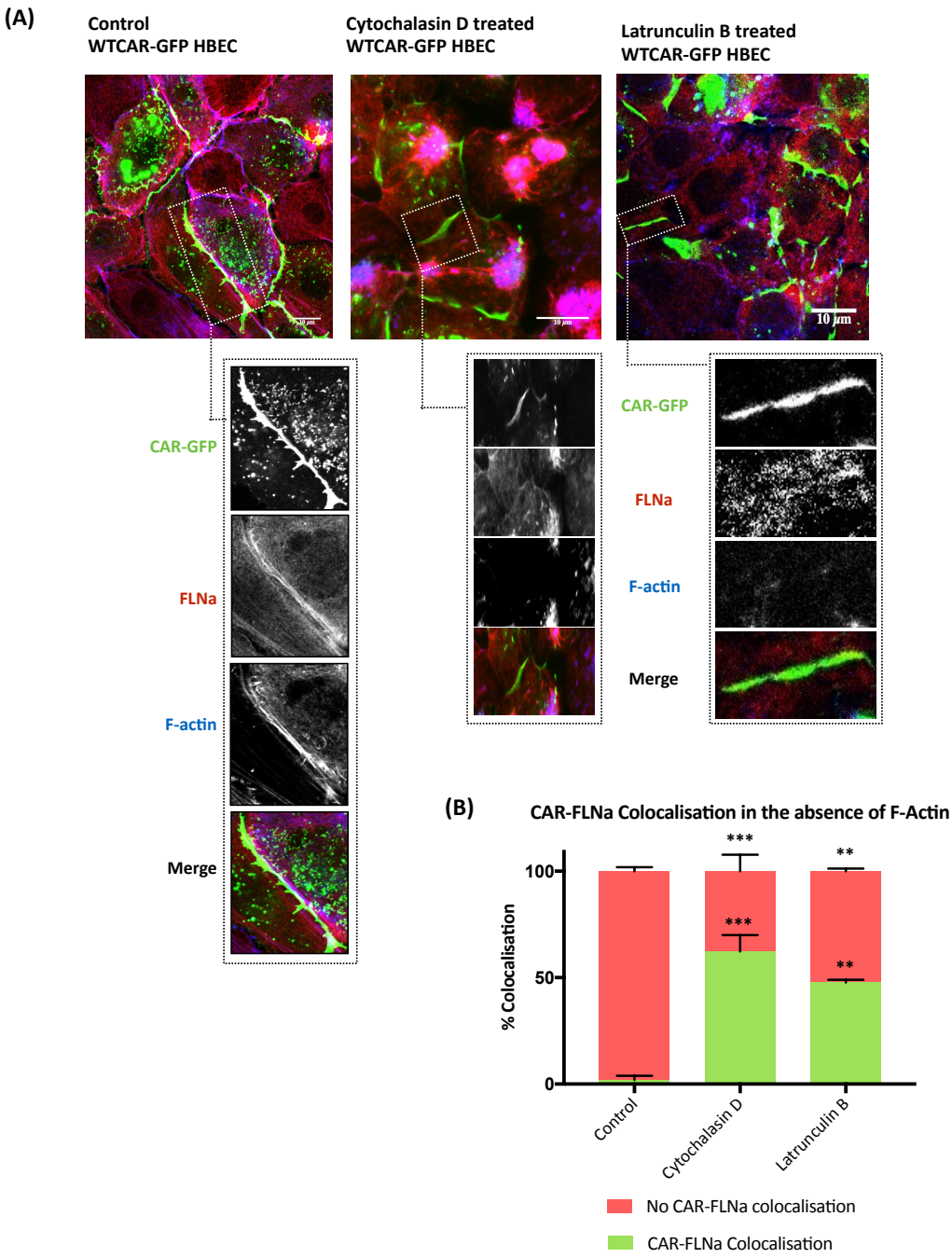
Next, the effect of F-actin disruption was studied on the ability of CAR to form a complex with FLNa. WTCAR-GFP HBECs treated with DMSO (control), Latrunculin B or Cytochalasin D were immunoprecipitated, analysed by western blotting and quantification of band intensity of FLNa was performed. A two fold reduction of CAR-FLNa complex was observed in cells treated with F-actin depolymerising agents (Fig.3.9A,B) suggesting that F-actin contributes to the formation of the CAR- FLNa complex.



3.9 **CAR-FLNa complex is reduced in cells treated with actin depolymerising agents.** (A) Immunoprecipitaion of endogenous FLNa from WTCAR-GFP HBECs treated with Cytochalasin D or Latrunculin B using GFP trap. Immunoprecipitates were separated by SDS-PAGE and analysed by immunoblotting for FLNa and GFP. GFP alone HBEC and WTCAR-GFP HBEC treated with DMSO were used as control. (B) Quantified band intensities of chemiluminescence blots from (A) of FLNa and GFP. FLNa was normalised against immunoprecipitated GFP. N=4, data are represented as mean±s.e.m. One-way ANOVA, Dunnett's, test, *= $p<0.05$, **= $p<0.01$, ***= $p<0.005$.

3.2.10. ***CAR-FLN complex is retained at F-actin disrupted cell:cell junctions***

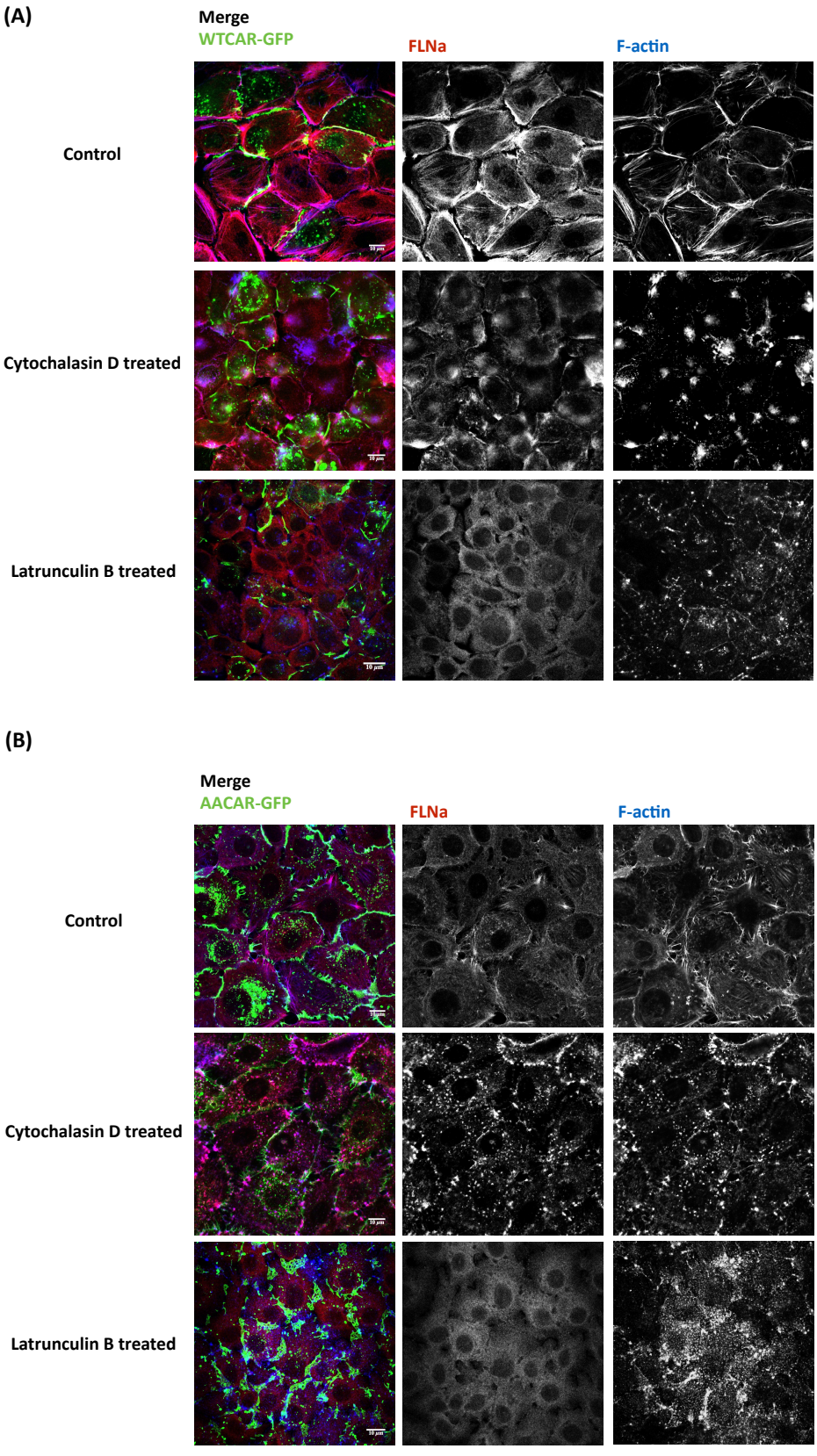
To check whether actin depolymerising agents would lead to loss of CAR-FLNa colocalisation at junctions, WTCAR-GFP HBECs were treated with DMSO (control), Latrunculin B or Cytochalasin D and immunostained for FLNa and F-actin. Confocal microscopy images revealed that CAR and FLNa colocalised at junctions even in the absence of F-actin lost due to Latrunculin B or Cytochalasin D treatment (Fig.3.10A). Percentage of CAR-FLNa colocalisation was quantified by a blind experiment. The percentage of CAR-FLNa colocalisation in the junctions lacking F-actin in DMSO treated cells was negligible (as all junctions had F-actin present) whereas 62.27% and 47.71% of Cytochalasin D and Latrunculin B treated cells showed CAR-FLNa complex at cell:cell junctions lacking F-actin (Fig.3.10B). This data confirms that while F-actin maybe required to facilitate initial CAR-FLNa binding, it is not required to maintain the CAR-FLNa complex stability.

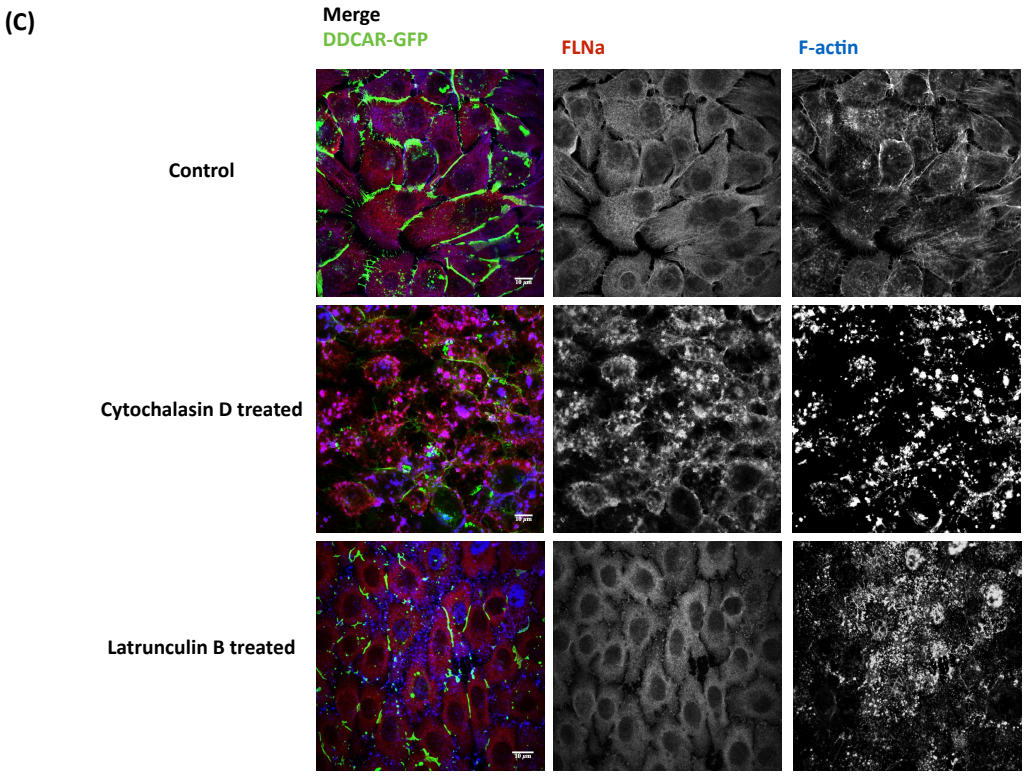


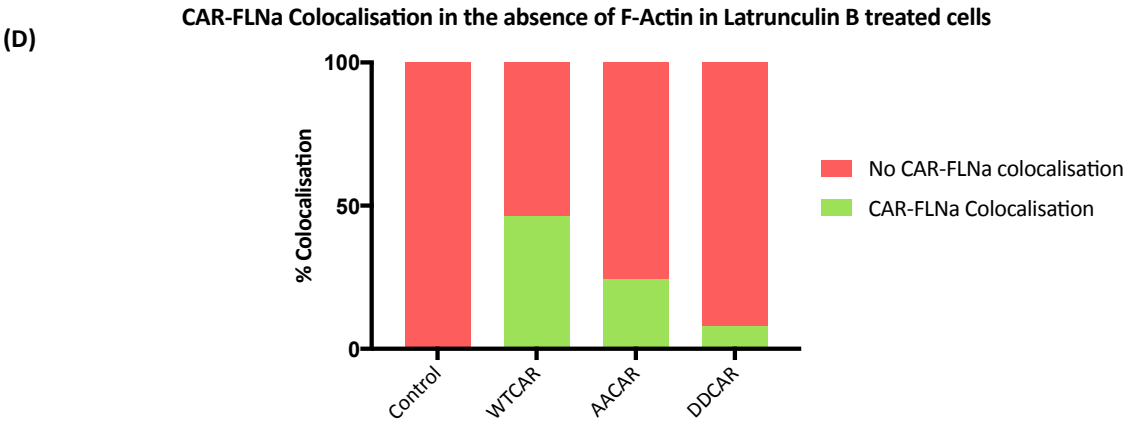
3.10. **CAR-FLN complex retained at F-actin disrupted cell:cell junctions.**(A) Confocal images of monolayers of control, Cytochalasin D or Latrunculin B treated WTCAR-GFP HBECs immunostained stained for FLNa (red) and F-actin (blue). Controls are DMSO treated. Scale bar : 10µm. (B) Histograms showing the percentage of colocalisation between CAR-FLNa at cell:cell junctions. N=2 independent experiment, n>60 cell:cell junction per condition, data are represented as mean±s.e.m. Two-way ANOVA, *= p<0.05, **= p<0.005, ***= p<0.0005.

3.2.11. ***Co-localisation of CAR-FLN is altered upon F-actin disruption***

Effect of F-actin depolymerising agents on CAR phosphomutants was analysed by repeating the experiment described in 3.3.10 on AACAR (Fig.3.11B) and DDCAR-GFP HBECs (Fig.3.11C). As shown in Fig 3.3.10, treatment with CytochalasinD resulted in retention of the WTCAR-FLNa complex (Fig.3.11A) but this was not the case in either of the phosphomutants. In case of AACAR-GFP cells, while AACAR was found to be retained at junctions that had lost F-actin, no FLNa was observed, potentially due to that fact that this mutant does not interact or colocalise with FLNa (Fig.3.3A,C & 3.5A). However, DDCAR was completely lost from junctions when cells were treated with CytochalasinD (Fig.3.11C) meaning no CAR-FLN colocalisation could be identified. Treatment of cells with Latrunculin B (a less harsh F-actin modulator), allowed for the quantification of percentage of colocalisation between CAR and FLNa at cell:cell junctions in a blind experiment. WTCAR- GFP cells treated with DMSO showed no colocalisation in junctions lacking F-actin (as all junctions contained F-actin), 46.42% of WTCAR-GFP cell junctions were found to have CAR-FLNa colocalisation in the absence of F-actin. 24.28% of AACAR and 7.42% of DDCAR-GFP cells showed CAR-FLNa colocalisation after being treated with Latrunculin B. Together this data suggests that phosphorylation of CAR may be important for CAR-FLNa complex formation and stability.


(A)
Merge
WTCAR-GFP
FLNa
F-actin
Control
Cytochalasin D treated
Latrunculin B treated
(B)
Merge
AACAR-GFP
FLNa
F-actin
Control
Cytochalasin D treated
Latrunculin B treated

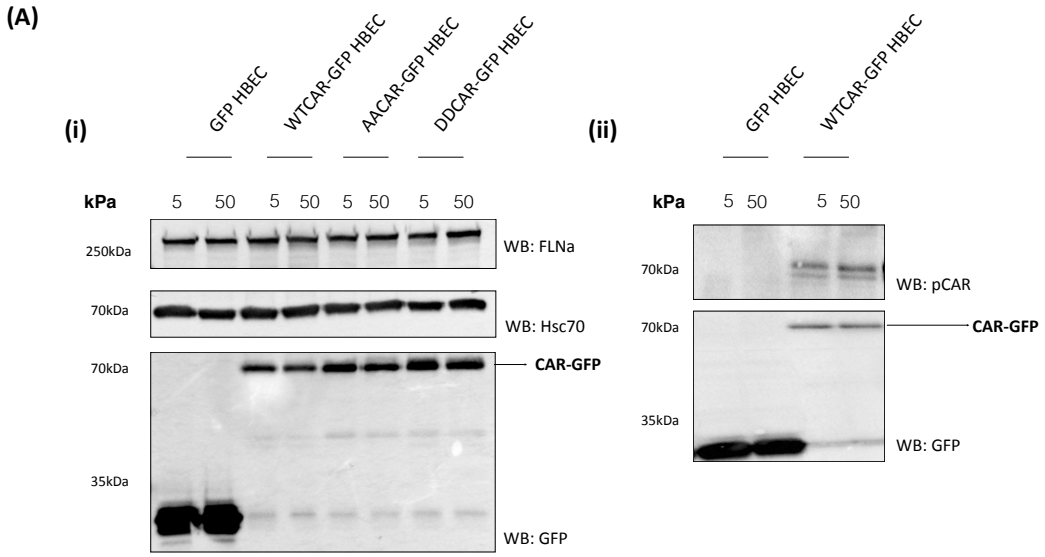


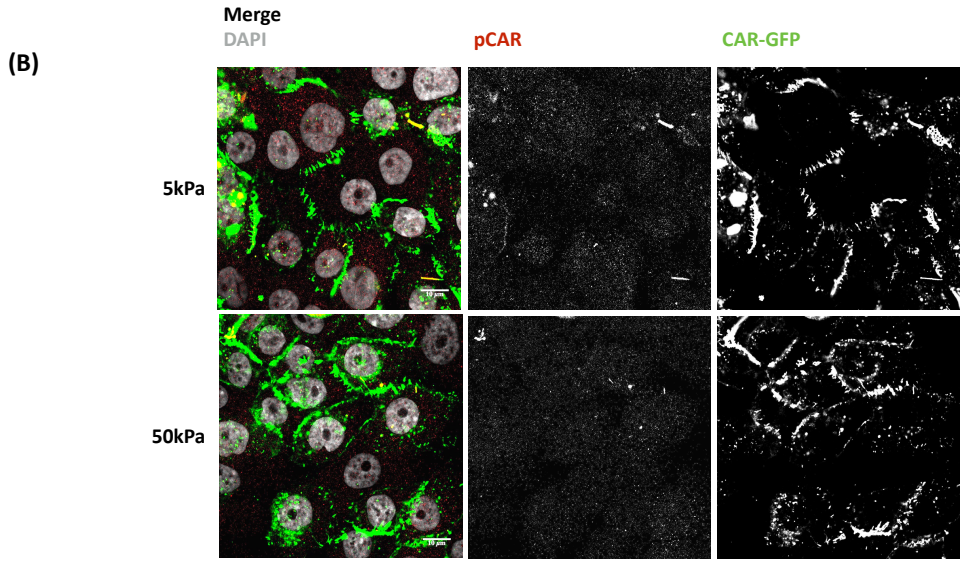


3.11. **Co-localisation of CAR-FLN is altered upon F-actin disruption.** Confocal images of monolayers of (i) control, Cytochalasin D or Latrunculin B treated (A) WTCAR-GFP HBECs, (B) AACAR-GFP HBECs or (C) DDCAR-GFP HBECs immunostained stained for FLNa (red) and F-actin (blue). Controls are DMSO treated. Scale bar : 10µm. (D) Histograms showing the percentage of colocalisation between CAR-FLNa at cell:cell junctions. N=1 independent experiment, n>60 cell:cell junction per condition, data are represented as mean.

3.2.12. ***FLNa colocalises with CAR at junctions in a phosphorylation dependent manner but does not change in response to altering substrate stiffness***

FLNa is a mechanosensor, actively detecting and responding to physical forces in the local environment (Nakamura et al 2007; Razinia et al 2012). Filamin A interactions with its partners such as FilGAP and beta-1-integrins are dictated by mechanical cues (Nakamura et al 2009; Liu et al 2015). To determine if external mechanical cues also play a role in the CAR-FLNa interaction, experiments were conducted on cells plated on PDMS gels of varying stiffness. Under physiological conditions, approximately 30kPa mimics *in vivo* tissue compliance of epithelial cells (Engler et al 2006). Therefore, stiffnesses of 5kPa and 50kPa were chosen to act as a soft and rigid substrate respectively. Western blot analysis of cells plated on the soft and rigid substrates showed similar levels of FLNa in WT HBECs, WTCAR, AACAR and DDCAR-GFP HBECs grown as monolayers in high calcium containing media (Fig. 3.12A(i)). Levels of phosphorylation of CAR also remained unchanged at 5 and 50kPa in WTCAR-GFP HBECs as revealed by western blot analysis (Fig. 3.12A(ii)). Confocal microscopy images of WTCAR-GFP cells confirmed similar phospho-CAR levels and showed increased FLNa colocalisation at junctions with phosphorylated CAR (Fig. 3.12B). Therefore, substrate stiffness does not alter the junctional localisation of phosphorylated CAR or expression levels of FLNa in WTCAR-GFP HBECs.

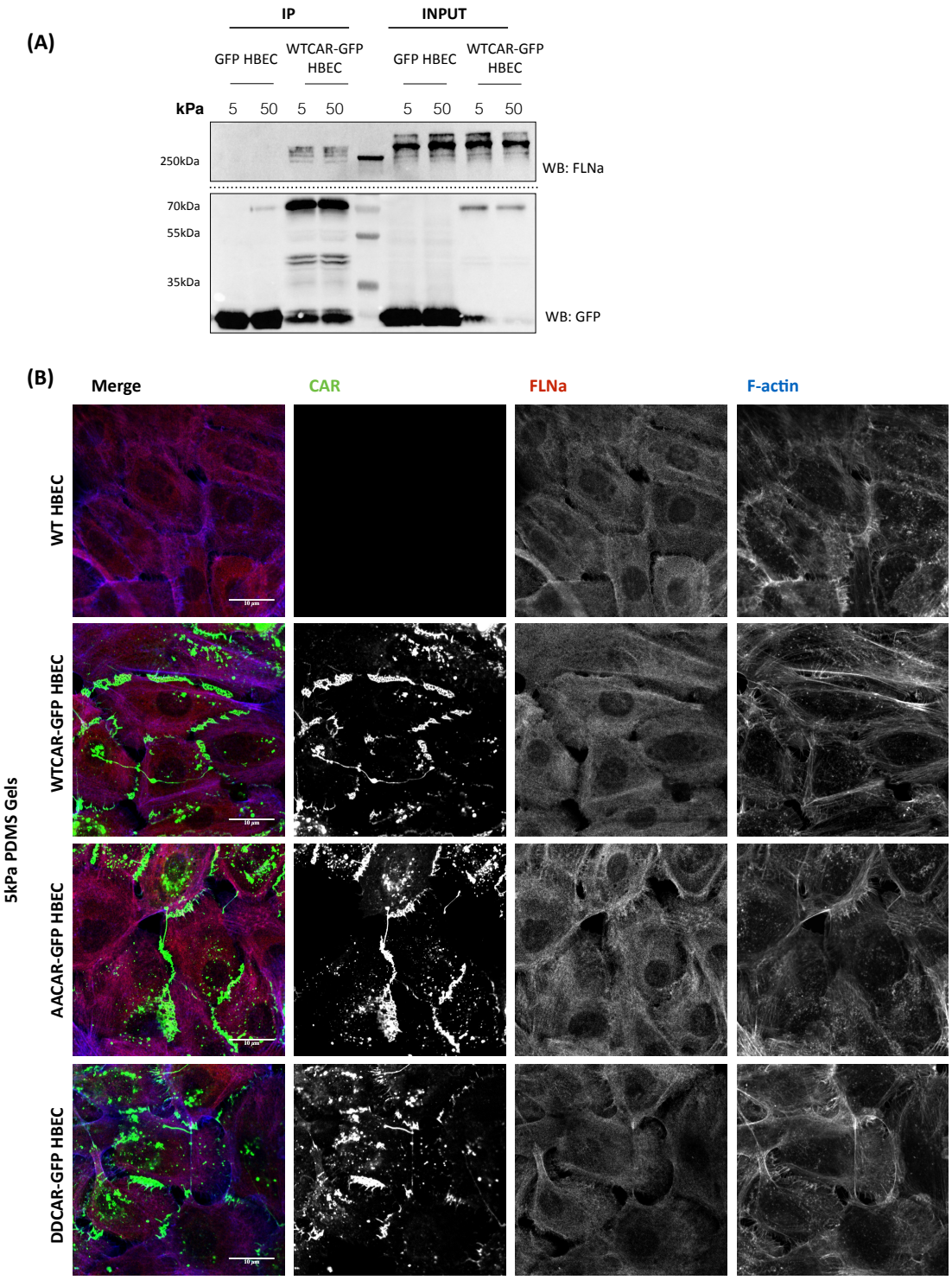


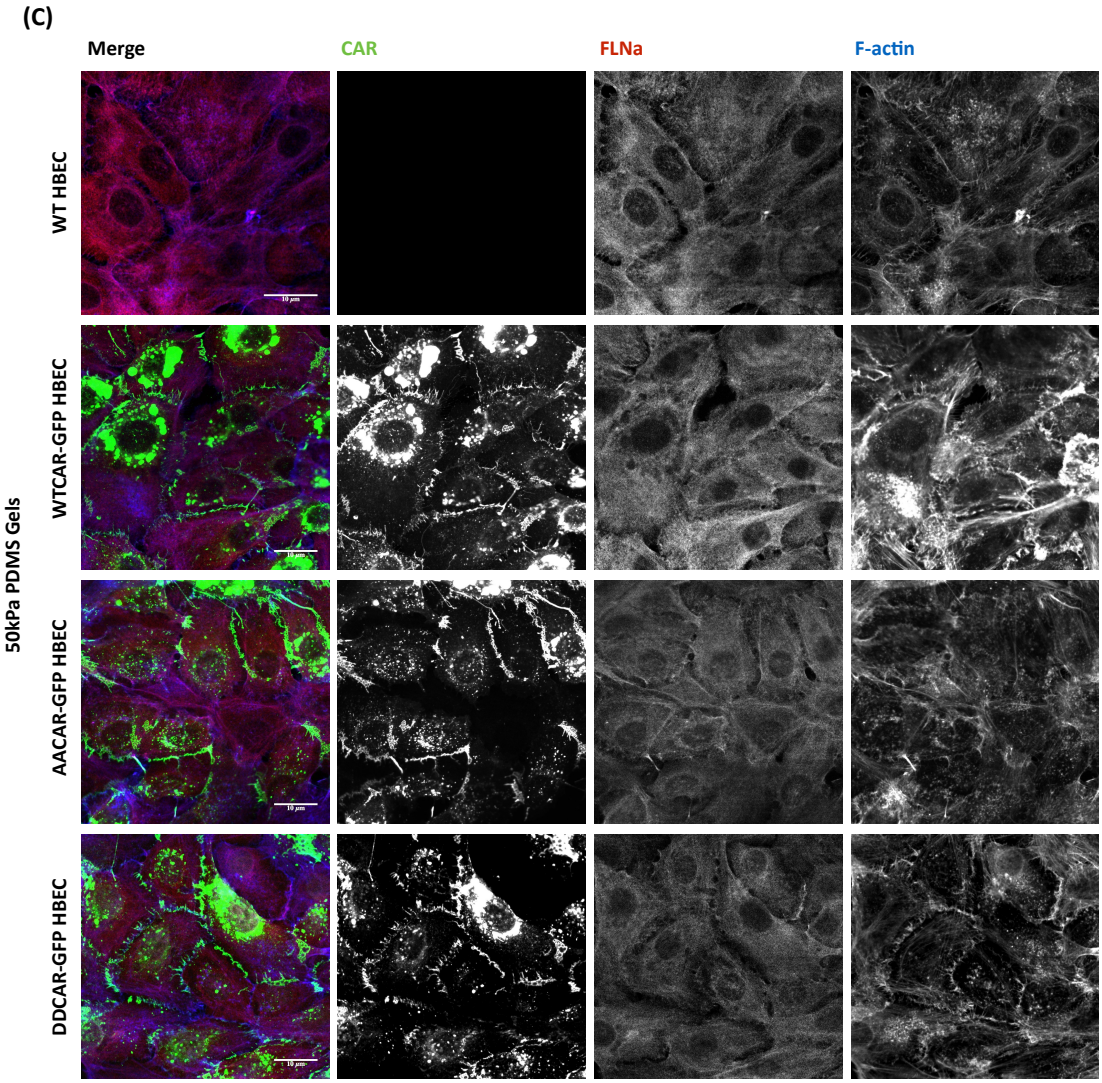


3.12. **FLNa colocalises with CAR at junctions in a phosphorylation dependent manner but does not change in response to different substrate stiffness.** (A) Western blot of lysates from WTCAR-GFP, AACAR-GFP or DDCAR-GFP HBECs grown on 5kPa or 50kPa PDMS gels and probed for FLNa, GFP or Hsc70 as loading control in (i) or pCAR and GFP as loading control in (ii). Cells were lysed with 2% SDS buffer inclusive of protease inhibitors and Calyculin A. (B) Confocal images of monolayers of WTCAR-GFP HBECs in calcium-containing media on 5kPa or 50kPa PDMS gels and immunostained stained for pCAR (red). Scale bar : 10µm.

3.2.13. ***Matrix stiffness does not alter CAR/FLN interactions***

To further investigate the effect of mechanical cues dictated CAR-FLNa interaction, WTCAR-GFP was immunoprecipitated and western blot analysed for FLNa from HBECs grown on soft and rigid substrates. Data revealed no changes in the levels of FLNa co-precipitated with CAR on different substrate stiffnesses (Fig.3.13A). The spatial distribution of FLNa was analysed by confocal microscopy in monolayers of WTCAR, AACAR and DDCAR HBECs. WT HBECs, used as controls, displayed dispersed FLNa with short cables of FLNa concentrated at the periphery of cells in both 5kPa and 50kPa PDMS gels. WTCAR and DDCAR showed FLNa terminating at punctuate CAR in junctions while AACAR had increased bundles of FLNa close to cell junctions on both substrate stiffnesses (Fig.3.13B,C). Therefore substrate stiffness does not appear to alter the spatial distribution of FLNa or regulate CAR- FLNa complex formation.


(A)
IP
INPUT
GFP HBEC
WTCAR-GFP HBEC
GFP HBEC
WTCAR-GFP HBEC
kPa
5
50
5
50
5
50
5
50
250kDa
WB: FLNa
70kDa
55kDa
35kDa
WB: GFP
(B)
Merge
CAR
FLNa
F-actin
5kPa PDMS Gels
WT HBEC
WTCAR-GFP HBEC
AACAR-GFP HBEC
DDCAR-GFP HBEC

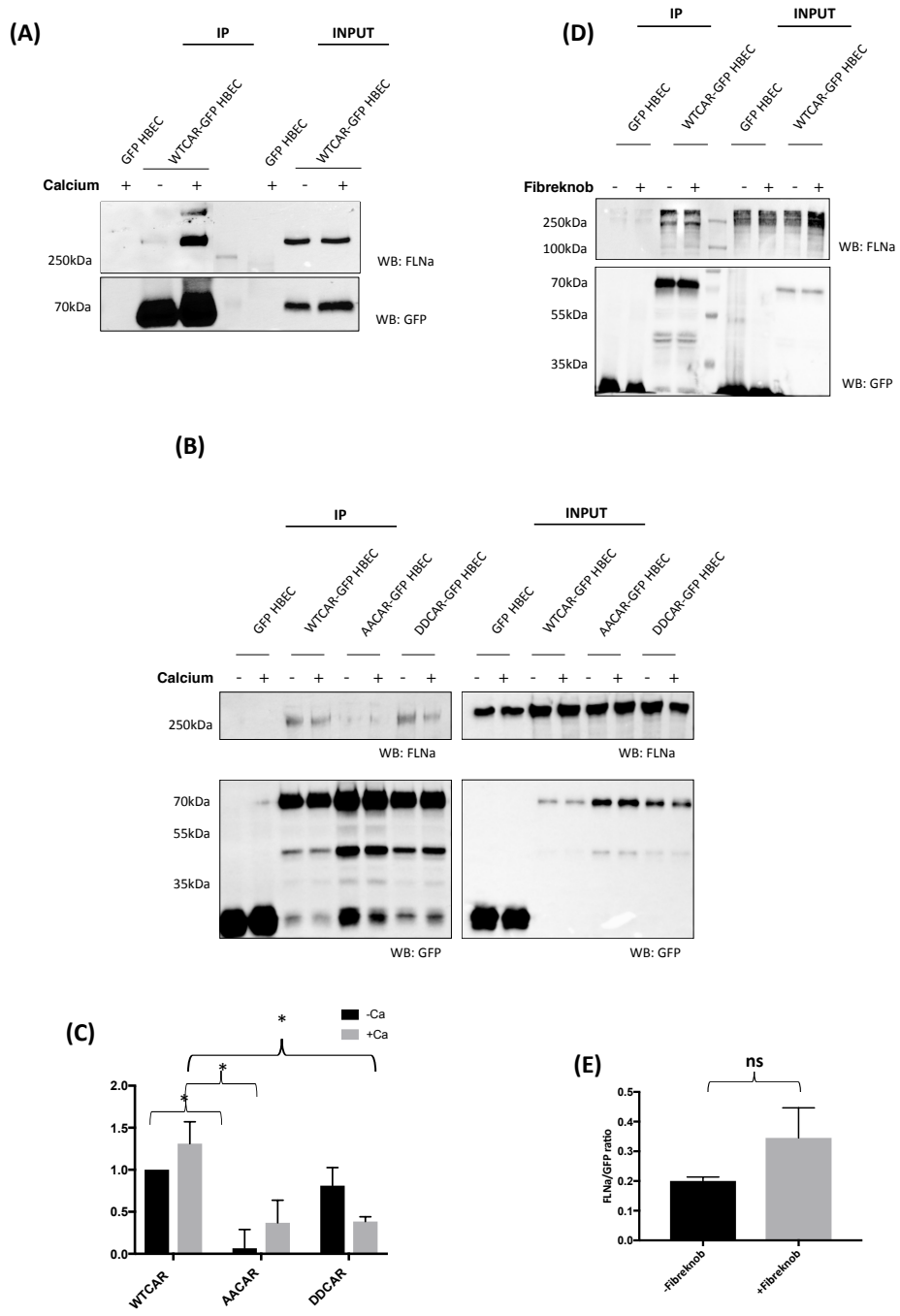


3.13. **Matrix stiffness does not alter CAR/FLN interactions.** (A) Immunoprecipitaion of endogenous FLNa from WTCAR-GFP HBECs grown on matrices of varying stiffness (5kPa or 50kPa) using GFP trap. Immunoprecipitates were separated by SDS-PAGE and analysed by immunoblotting for FLNa and GFP. GFP alone HBECs were used as control. Confocal images of monolayers of WTCAR-GFP, AACAR-GFP or DDCAR-GFP HBECs grown in calcium-containing media on (B) 5kPa or (C) 50kPa PDMS gels and immunostained stained for FLNa (red) and F-actin (blue). Scale bar : 10µm.

3.2.14. ***CAR-CAR homodimerisation promotes FLNa binding***

CAR homodimerises *in trans* at cell:cell junctions (Coyne, & Bergelson 2005). However, whether CAR homodimerisation plays a role to play in its intracellular functions or in binding to intracellular proteins remains unclear. We have consistently observed CAR-FLNa colocalisation only at cell:cell junctions and therefore hypothesised that the CAR-FLNa interaction may require CAR to homodimerise across cell junctions. WTCAR- GFP HBECs grown as single cells or in confluent monolayers under calcium rich conditions (to promote stronger cell junctions) showed a greater affinity of FLNa to bind CAR when CAR was present as homodimers at cell junctions (Fig.3.14A). Monolayers of WTCAR, AACAR and DDCAR-GFP HBECs grown with or without calcium did not show a clear difference in their binding to FLNa (Fig.3.14B,C). However, an reduction in the DDCAR-FLNa complex in cells grown as monolayers was observed as compared to WTCAR HBECs grown under the same conditions (Fig.3.14C). Taken together, this data reveals a role for CAR-CAR interaction at cell junctions in the CAR-FLNa complex formation.

To further test the importance of CAR homodimerisation in CAR's ability to bind FLNa, we disrupted CAR homodimers by addition of the Adenovirus type 5 fibreknob (FK) domain. Ad5FK binds to CAR with higher affinity than CAR binds to itself, thereby disrupting its homodimerisation (Lortat-Jacob et al 2001). WTCAR-GFP was immunoprecipated from HBECs treated with or without FK protein for 4 hours. No significant difference was observed in binding of CAR to FLNa following FK treatment (Figure 3.14D, E).



3.14. **CAR-CAR homodimerisation promotes FLNa binding.** Immunoprecipitation of endogenous FLNa from (A) WTCAR-GFP HBECs grown as sparse cells or confluent monolayers in the presence of calcium, (B) WTCAR-GFP, AACAR-GFP or DDCAR-GFP HBECs grown with or with calcium or (D) confluent monolayers of WTCAR-GFP HBECs treated with or without fibreknob protein, using GFP trap. Immunoprecipitates were separated by SDS-PAGE and analysed by immunoblotting for FLNa and GFP. GFP alone HBECs were used as control. (C) Quantified band intensities of chemiluminescence blots from (B) of FLNa and GFP FLNa was normalised against immunoprecipitated GFP. N=4, data are represented as mean±s.e.m. One-way ANOVA, Dunnett's. (E) Quantified band intensities of chemiluminescence blots from (D) of FLNa and GFP FLNa was normalised against immunoprecipitated GFP. N=3, data are represented as mean±s.e.m. T-test, ns= not significant, *= p<0.05, **= p<0.01, ***= p<0.005.

3.3. **Discussion**

CAR is well established as a tight and adherens junction component, but very little is known about its recruitment, binding partners or signalling effects at cell junctions. This chapter aimed to characterise and validate Filamin A, an F-actin cross linker, as a novel binding partner of CAR.

We have previously shown that CAR localised to cell:cell junctions regulates E-cadherin dynamics and influence integrin activation (Hussain et al 2011; Farmer et al 2009). FLNa plays an important role in cell:matrix signalling and in association with F-actin is a key scaffolding protein for many signalling molecules, especially the integrin adhesion receptors (Stossel et al 2001; Popowicz et al 2006). Filamin A plays a number of different roles from facilitating localisation and transport of receptors to F-actin remodelling which is crucial for cell survival, membrane protrusion and adhesion/migration (Nobes et al 1995; Meng et al 2004; Bellanger et al 2000; Vadlamudi et al 2002). Data presented here confirms the binding of CAR phosphorylated at Thr290/Ser293 and FLNa. The cellular distribution of FLNa was not only found altered but that its association with phosphorylated CAR may prevent FLNa from binding to itself or other proteins on neighbouring cells. This is evident from the loss of intercellular FLNa extensions at CAR junctions in WTCAR and DDCAR cells (Fig.3.3).

Our previous data has suggested phosphorylation of CAR cytoplasmic tail is controlled through protein kinase C delta (PKCδ), which may act as the critical kinase that regulates this interaction (Morton et al 2013). This data, however, only confirm that ability of CAR-FLNa to form a complex but does not establish whether these proteins can directly bind to each other. Direct analysis of protein-protein

interactions using methods such as yeast two-hybrid, purified recombinant protein interactions in vitro or fluorescence resonance energy transfer (FRET) between CAR-GFP and FLNa-mRFP would potentially address this, as well as potentially providing novel insight into when and where this complex is assembled in epithelial cells in monolayers.

Using GST pulldowns, data shown here demonstrates that CAR binds potentially to multiple domains on FLNa, but with the greatest affinity for the Actin Binding Domain (ABD), which plays an important role in determining the localisation of FLNa to F-actin structures (Washington, & Knecht 2008). Data presented here shows that CAR also binds to FLNa-ABD1-15, which includes the Rod 1 region that has low-affinity binding to F-actin. Data in the report also shows a marked reduction in CAR-FLNa interaction upon disruption of F-actin cytoskeleton. These indicate a role for the CAR cytoplasmic domain to locally stabilise F-actin stress fibres and / or the possibility of a direct interaction with F-actin. Overexpression of ABD increased the CAR-FLNa interaction. When expressed in filamin deficient cells, ABD can localise and bind to actin, thereby, suggesting that it acts independently and in fact regulates filamin's ability to associate with actin (Washington, & Knecht, 2008). It is, therefore, possible that the overexpressed ABD replaces or displaces the ABD in endogenous FLNa from binding to F-actin and thereby promotes the latter associating with CAR. Another possibility is the potential ABD homodimerisation with endogenous FLNa, which can promote FLNa-CAR complex formation. It is not uncommon for actin binding sites to dimerise, for example, in utrophin and dystrophin (Keep et al 1999; Norwood et al 2000). Furthermore, the manner in which the ABD dimerises directly impacts on the protein's function (Norwood et al 2000). It would therefore be very interesting and relevant to initially test FLNa-ABD

dimerisation through simple experiments such as size exclusion chromatography. The ABD-ABD interaction can then be confirmed through more reliable techniques such as Bimolecular Fluorescence Complementation or Förster Resonance Energy Transfer.

Interestingly, the Rod2 domain of FLNa is also shown to bind CAR. Previous studies have demonstrated that binding of IgFLNa21 (which is within the Rod2 domain) to β1 integrin tail inactivates the integrin, which has an impact on cell:matrix adhesion turnover and signalling (Loo et al 1998; Kiema et al 2006; Calderwood et al 2001). Our previous studies have shown CAR activates β1 integrin through MAPK induction (Farmer et al 2009) suggesting that CAR may act as a molecular switch in integrin activation through its engagement with FLNa. We explore this possibility further in the next chapter.

It is also striking to note that FLNa mostly localises with CAR at cell:cell junctions and shows no colocalisation but very little overlap in single cells. CAR homodimerises *in trans* at cell:cell junctions (Coyne, & Bergelson 2005). Our experiments in high calcium conditions increase *trans* junctional interactions of E-cadherin thereby forming more stable junctions (Kim et al 2011a). This in turn promotes greater CAR:CAR interaction at cell-cell adhesion sites. Under these conditions, FLNa also binds to CAR at far higher levels than in single cells, strongly implying that CAR homodimerisation may have a role to play in its ability to engage with FLNa. We further experimented this assertion by disrupting CAR homodimerisation with adenovirus 5 fibre knob (Lortat- Jacob et al 2001). Although the difference was not significant, addition of fibre knob showed a trend towards a slight increase in CAR-FLN binding. This seems counter-intuitive; however, data presented in this chapter

demonstrates that high levels of phosphorylated CAR can be detected by immunostaining at cell-cell adhesions that are under tension or undergoing apparent disassembly. This may suggest that the presence of the FK may increase phosphorylation of CAR and this may increase FLN binding. Longer time course treatments of HBECs with fibre knob would determine whether this reagent could disrupt CAR-FLNa interactions after junctions had disassembled fully. Analysis of CAR, pCAR and FLN colocalisation in FK treated cells over a time course of treatment would also help to define whether this hypothesis holds true.

An additional important observation was made in this chapter that phospho-mutants of CAR display varying degrees of stability during the loss of F-actin (Fig. 3.11). While WTCAR and AACAR were retained at actin-disrupted junctions, DDCAR was completely lost upon Cytochalasin or Latrunculin treatment. This suggests that non-phosphorylated CAR may have a greater ability to remain associated at junctions as homo-dimers compared to non-phosphorylated CAR. This would further imply that differential phosphorylation of CAR may control stability and movement of CAR at junctions and perhaps alter junctional dynamics. These concepts are explored further experimentally in the next results chapter.

Given that FLNa is well established mechanosensor and transducer, and that a number of junctional proteins have been implicated in mechanosensing roles, we hypothesised that mechanical cues may influence CAR-FLNa interaction. In this study, we used soft (5kPa) and rigid (50kPa) substrate poly-dimethylsiloxane (PDMS) gels to provide external mechanical cues to HBECs but found no alteration in the expression levels or colocalisation patterns of CAR and FLNa. Under physiological conditions, epithelial cells experience 30kPa rigidity. Perhaps, increasing stiffness to

higher values that mimic disease or abrasive conditions might uncover more defined phenotypes.

In conclusion, data shown here provides evidence that Filamin A as novel binding partner of CAR that may influence the role of CAR in cell:cell and cell:adhesion assembly, paving way for future studies in adhesion and migratory signalling under homeostatic and inflammatory conditions in epithelial cells.

4. CAR Regulates Epithelial Cell Adhesion & Migration

4.1. **Introduction**

CAR is an important player mediating cell:cell adhesions and cell migration (Cohen et al 2001; Walters et al 2002; Vincent et al 2004; Wang et al 2007 Caruso et al 2010; Morton et al 2013). CAR has also been previously shown to regulate migration in U87 glioma cells via the binding of tubulin to the cytoplasmic tail of CAR (Fok et al 2007). CAR has been shown to contribute in F-actin binding and more recently, to interact with Rho-associated protein kinase (ROCK), an F-actin regulator (Huang et al 2007; Saito et al 2014). It also binds to a number of other proteins through the C-terminal domain including TJ proteins PICK1, MUPP1 and ZO-1 (Cohen et al 2001; Coyne et al 2004; Excoffon et al 2004). As well as binding partners, the C-terminal domain has also been shown to regulate intracellular signalling cascades. Previous studies have shown a role for CAR in activation of p44/42 MAPK and JNK signalling pathways in different cell types (Cunningham et al 2003; Tamanini et al 2006; Marchant et al 2009). Work from our laboratory has also shown that CAR can control epithelial cell adhesion through activation of p44/42 MAPK (Farmer et al 2009). Increased activation of β1 and β3 integrins, that are essential for cell:matrix adhesion in epithelial cells, was found to be a direct consequence of CAR-induced p44/42 activation. It was also demonstrated that the cytoplasmic domain of CAR is required for p44/42 activation, integrin activation and CAR's localisation to cell junctions (Farmer et al 2009). Also, data from our lab and others has shown that overexpression of CAR can disrupt the recruitment and localisation of E-cadherin to epithelial cell:cell junctions (Hussain et al 2011; Caruso et al 2010). These studies suggest that CAR can potentially act to co-ordinate the

signalling between cell:cell and cell:matrix adhesion sites to maintain epithelial cell integrity.

Data presented in the previous chapter demonstrated that Filamin A (FLNa) is a novel binding partner for CAR. FLNa is a well-established mediator of cell adhesion and migration. Studies have shown that the loss of FLNa (and FLNb) impairs initiation of cell migration (Baldassarre et al 2009). In HT1080 cell lines, Baldassarre et al (2009) observed that migration defects in filamin double knockdowns (FLNabKD), although recused by re-expressing full length FLNa, could not be rescued by FLNΔ19-21 (present in the rod2 region). This suggests that the ABD region and the IgFLN19-21 are essential for the initiation, if not the entire process, of cell migration. The β chain cytoplasmic tails of clustered integrins bind onto the IgFLN21 repeats on the FLNa dimers (Pfaff et al 1998; Chen et al 2013; Nakamura et al 2007). Together FLNa-β1 complex functions as a link between the ECM and cytoskeletal actin, thereby mediating bidirectional cell signalling, formation of transient membrane protrusions and cell polarisation (Calderwood et al 2001; Gehler et al 2009). The tight binding of FLNa negatively regulates integrin mediated migration (Calderwood et al 2001). Migfilin, a filamin binding protein found in cell:cell and cell:matrix adhesions, displaces filamin A from β1 integrin, thus, acting as a molecular switch for β1 integrin activation (Das et al 2011; Ithychanda et al 2009).

Experiments in this chapter were aimed at exploring the possibility of CAR-FLNa interaction in regulating the activation status of β1 integrins, and subsequently epithelial cell migration. The potential role for CAR in regulating cell:cell and cell:matrix adhesions and mediating a crosstalk between the two adhesive sites are also explored.

The aims of this chapter were:

- To determine the role of CAR in controlling epithelial cell focal adhesion assembly.
- To examine the importance of CAR-FLNa in reactivating β1 integrin
- To study the role of CAR in maintaining epithelial cell junction stability
- To study the role of CAR in epithelial cell migration
- To determine CAR's role in co-ordinating the signalling between cell:cell and cell:matrix adhesion sites

4.2. **Results**

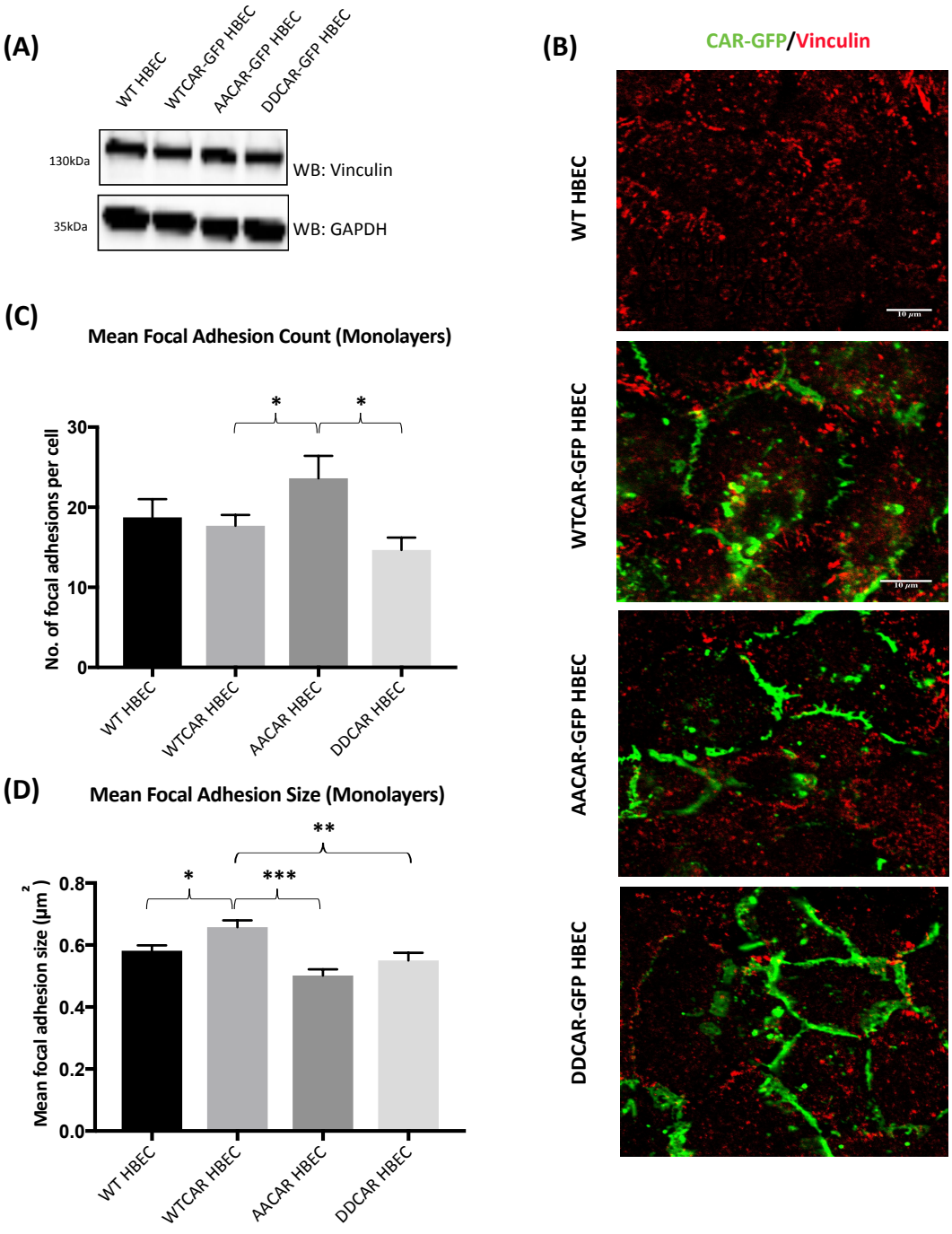
4.2.1. ***CAR regulates focal adhesion number in HBEC***

Mechanical coupling between the actin cytoskeleton and the extracellular matrix is crucial for cell architecture, adhesion, signalling and migration (reviewed in Wehrle-Haller 2012). Focal adhesions (FA), which form this mechanical link, are rich in activated integrins that form clusters. Integrins bind to extracellular ligand and relay signals to the actin cytoskeleton (outside-in signalling) through recruitment of a number of cytoplasmic adaptor proteins such as talin and vinculin (Hynes 2002; Wehrle-Haller 2012). These adaptor proteins are able to bind directly to the F-actin cytoskeleton or other adhesion-associated proteins to promote focal adhesion stability. To determine whether CAR might regulate focal adhesion phenotypes, HBEC or those expressing CAR-GFP forms were first assessed for levels of total vinculin to determine whether this might act as a valid marker for this analysis. Expression levels of vinculin protein as assessed by western blotting remained the same across all HBEC (Fig.4.1A) and therefore was used as a marker for FA to analyse the role of CAR in controlling FA assembly.

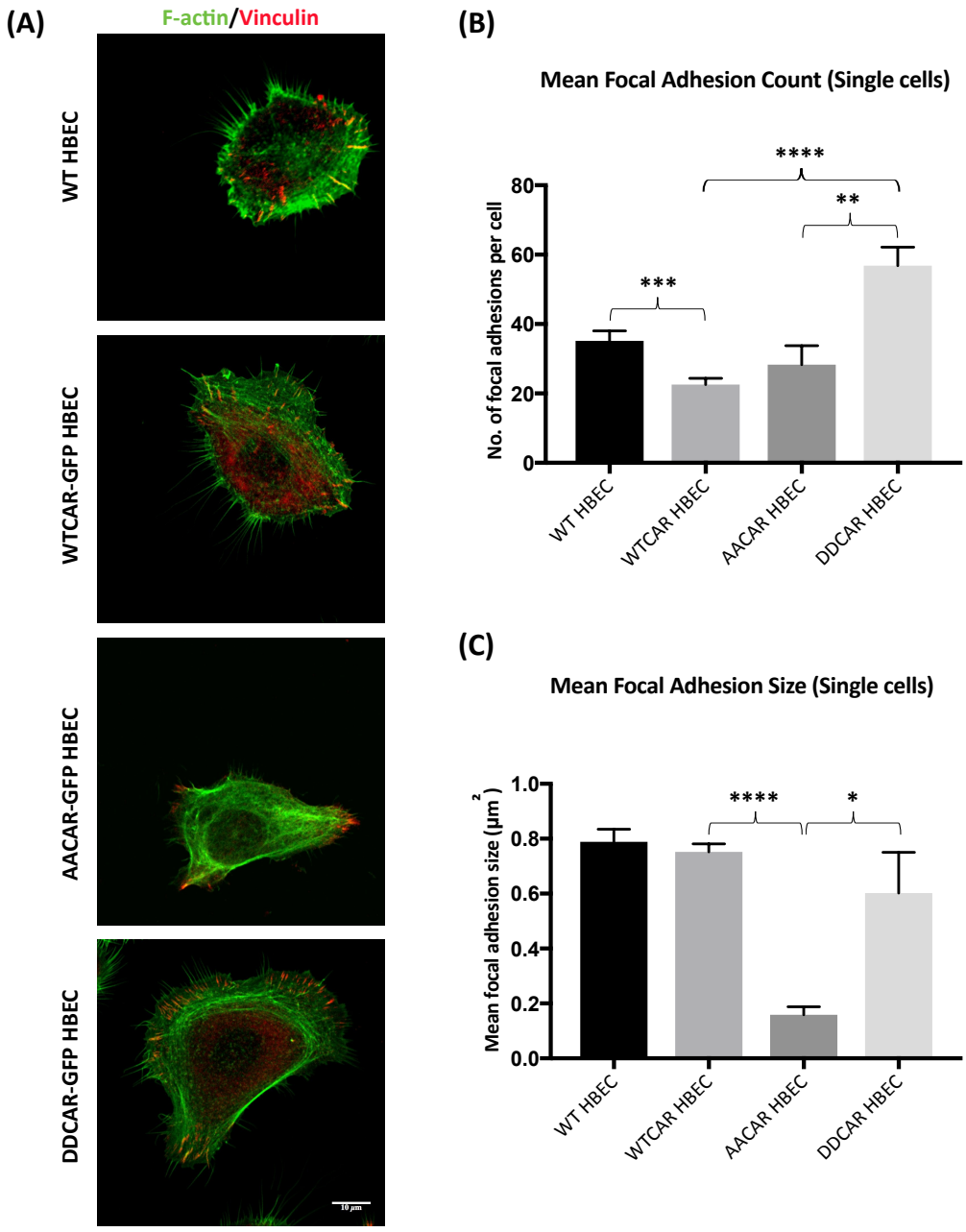
Since FLNa associated with phosphomutant CAR (Fig.3.5) we sought to investigate the behaviour of FA in cells expressing phospho-mimic and non-phosphorylated mutants of CAR. Parental (WT), WTCAR-GFP, DDCAR-GFP and AACAR-GFP HBEC were grown as monolayers in high calcium, fixed and immunostained with vinculin and imaged by confocal microscopy (Fig. 4.1B). Focal adhesions were found to be concentrated beneath CAR-enriched

junctions in the WTCAR-GFP and DDCAR-GFP cells, suggesting that phosphorylated CAR may contribute towards sub-cellular localisation of adhesion assembly. In contrast, FA localisation in control HBEC and AACAR-GFP HBEC appeared more dispersed across the base of the cell. Quantification of FA number in HBEC grown as monolayers (in the presence of calcium to promote cell:cell junction formation) or as single cells (in the absence of calcium) was performed to assess the number and size of focal adhesions from confocal images of vinculin stained populations.

In monolayers, numbers of vinculin-containing FA in WTCAR-GFP HBEC remained the same as control (WT) HBEC but mean FA size was increased (Fig.4.1C,D). In contrast, AACAR-GFP HBEC showed an increase in the number of FAs in comparison to WT and WTCAR HBEC but there was no change in FA in DDCAR HBEC compared to WT cells (Fig.4.1C). However, both AACAR and DDCAR cells showed significantly smaller FA when compared to WTCAR cells (Fig.4.1D). Under sparse, single cell conditions (Fig4.2A), WTCAR HBEC assembled fewer FA of similar to size to that of control WT HBEC (Fig.4.2B,C). AACAR HBEC had fewer, smaller FA and DDCAR HBEC had more, larger FA when compared to WTCAR HBEC (Fig4.2B,C). These data combined suggest that CAR plays a role in controlling FA assembly and stabilisation in cells, and that this depends on the phosphorylation status of the CAR cytoplasmic tail.



4.1 **CAR does not alter vinculin protein levels in HBEC monolayers but alters Focal Adhesion (FA) number.** (A) Western blot of lysates from wilt-type (control), stable CARGFP and and its phospho-mutants overexpressing HBECs probed for Vinculin or GAPDH as loading control. Cells were lysed with 2% SDS buffer inclusive of protease inhibitors and Calyculin A. (B) Confocal images of monolayers of HBECs over-expressing GFP-tagged FL-CAR and its cyto-tail mutants grown in calcium-containing media and immunostained for Vinculin (red). Histograms of (C) average focal adhesion count normalised to cell numbers and (D) average focal adhesion size in monolayers of HBECs. N= 2 independent experiments, n ≥ 20 cells per cell type. Data are represented as mean±s.e.m. One-Way ANOVA *= p≤0.05, **= p≤0.005, ***= p≤0.0005, ****= p≤0.0001. Scale bar : 10µm.



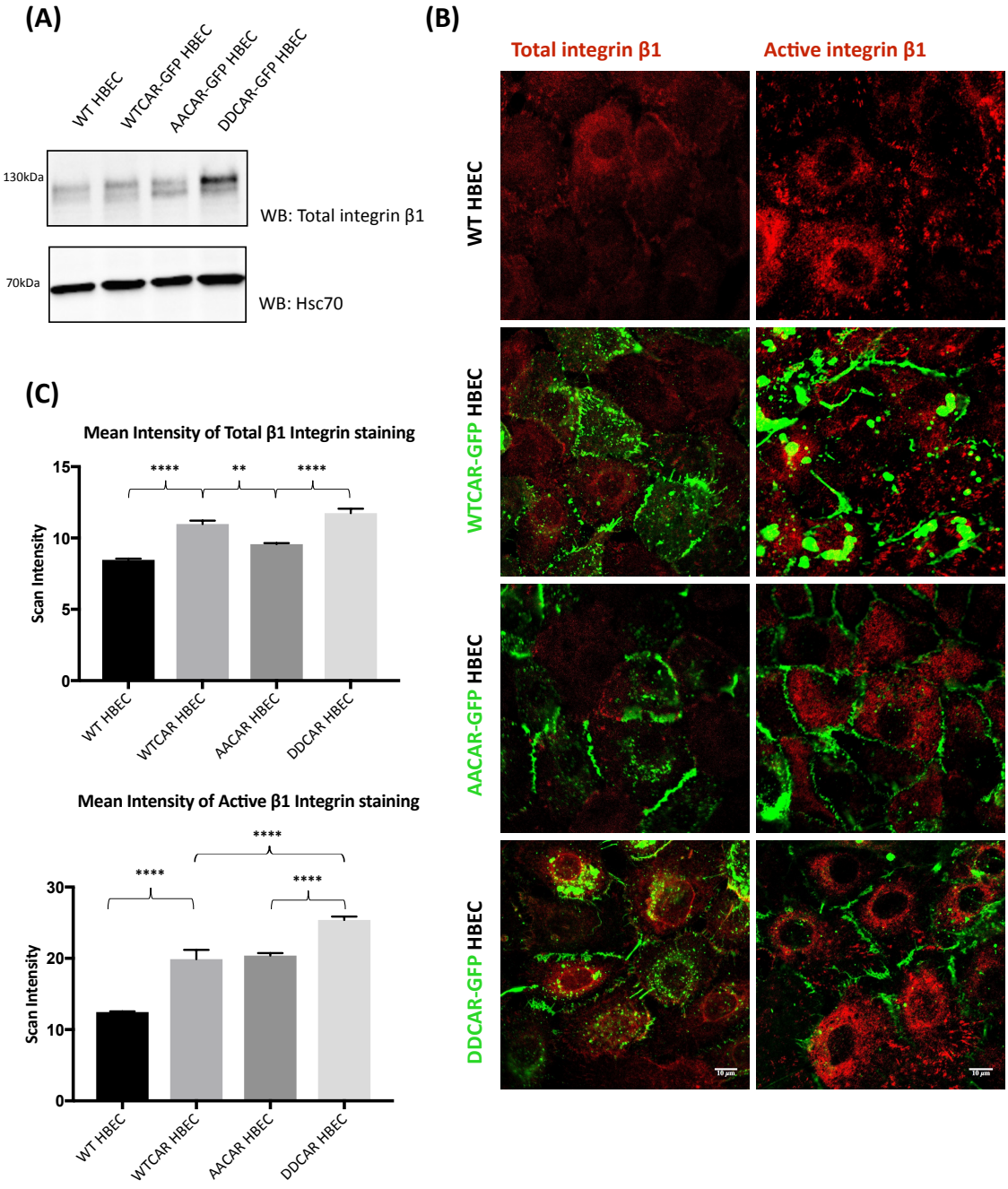
4.2 **CAR does alters vinculin containing Focal Adhesion (FA) number in single HBEC.** (A) Confocal images of single HBECs over-expressing WT-CAR and its cyto-tail mutants grown in calcium-containing media and immunostained for Vinculin (red) and F-actin (green). Histograms of (F) average focal adhesion count per cell and (G) average focal adhesion size in single HBECs. N= 2 independent experiments, n ≥ 20 cells per cell type. Data are represented as mean±s.e.m. One-Way ANOVA *= p≤0.05, **= p≤0.005, ***= p≤0.0005, ****= p≤0.0001. Scale bar : 10µm.

4.2.2. ***The phosphorylation status of CAR alters integrin activation in HBEC***

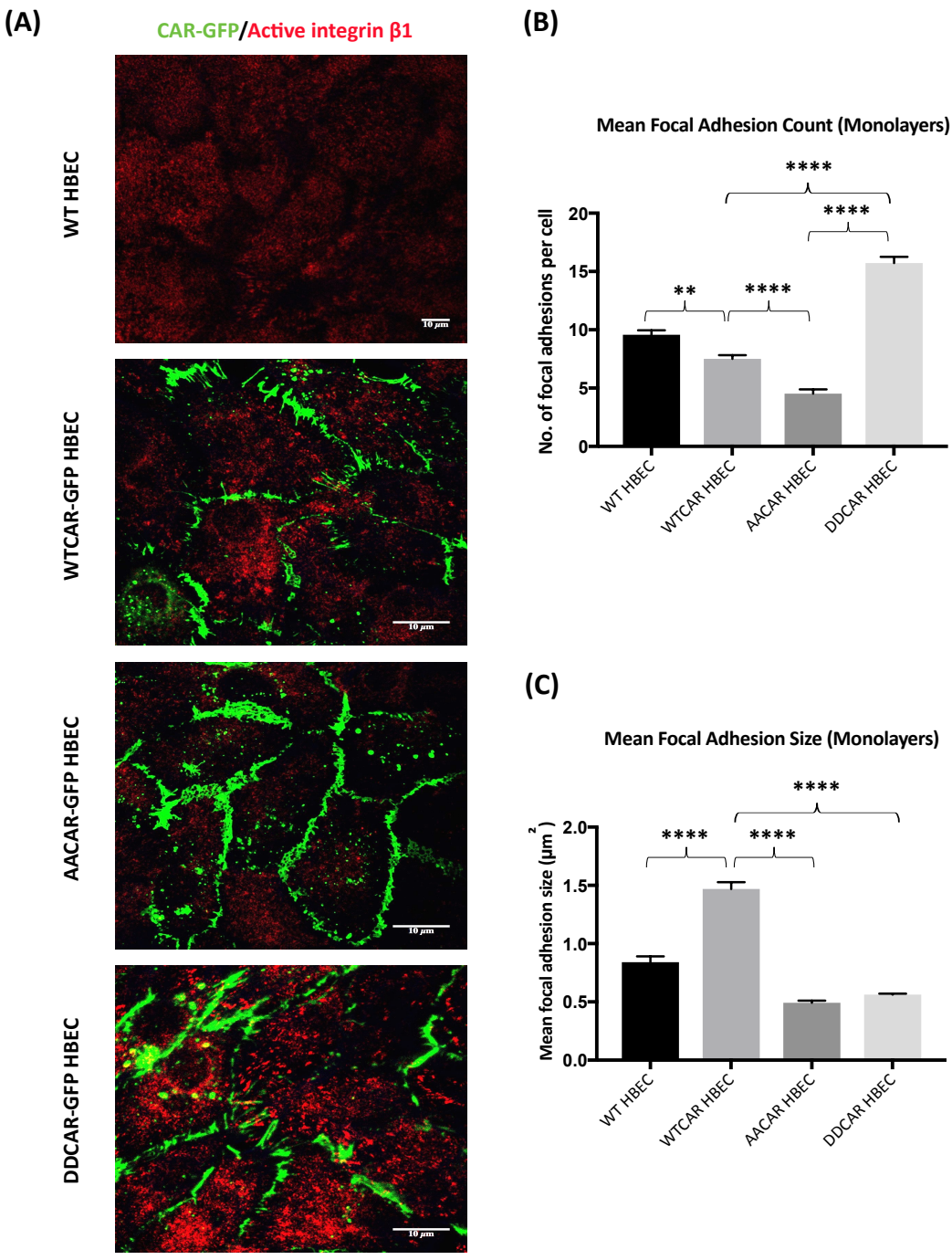
To investigate the spatial relationship between β1-integrin and CAR, the localisation of total and activated populations of β1 integrin was analysed in HBEC. Cells were plated in high calcium media to promote cell:cell junction formation and analysed for total β1 integrin by western blotting. Total β1 integrin levels varied across the cell lines between experiments without any consistent trend towards changes between the cell lines (Fig4.3A). The double bands observed are likely to be due to differences in post-translational modifications of the protein. Total or active β1 integrin populations were then analysed in the same cell lines by immunostaining (Fig 4.3B). Activated β1 integrin mostly localised to focal adhesions near to or beneath cell junctions and internal pools of the cell, while total β1 integrin was seen at both focal adhesions and cell:cell junctions, the latter most prominently in WT HBEC and to a lesser extent in WTCAR and DDCAR cells. In AACAR cell however, total β1 integrin was found dotted along or close to cell junctions. Strikingly, AACAR cells had very few FA with active β1 integrin. Quantitation of the intensity of staining for total and active β1 integrin was then carried out to compare between cells. Analysis revealed higher intensity staining of total β1 integrin in WTCAR and DDCAR expressing cells compared to WT HBEC, but not AACAR cells (Fig 4.3C). Activated β1 integrin intensity was also significantly increased in WTCAR and DDCAR cells compared to WT HBEC, but in this case AACAR cells also showed an increase, although localisation of the active integrin was more diffusely distributed (Fig.4.3C). To determine whether the changes in intensity of active β1 integrin was due to active integrin recruitment into adhesions, FA number and size were

quantified in HBEC stained with active β1 integrin antibodies (Fig 4.4A). Quantification revealed that WTCAR HBEC had fewer but larger FA compared to control cells with an overall reduction in number and size of FA in AACAR cells but more, smaller FAs in DDCAR compared to WTCAR cells (Fig.4.4B,C).

Taken together, these data strongly suggest that phosphorylated CAR can enhance the number of focal adhesions and activation of β1 integrins in HBEC.



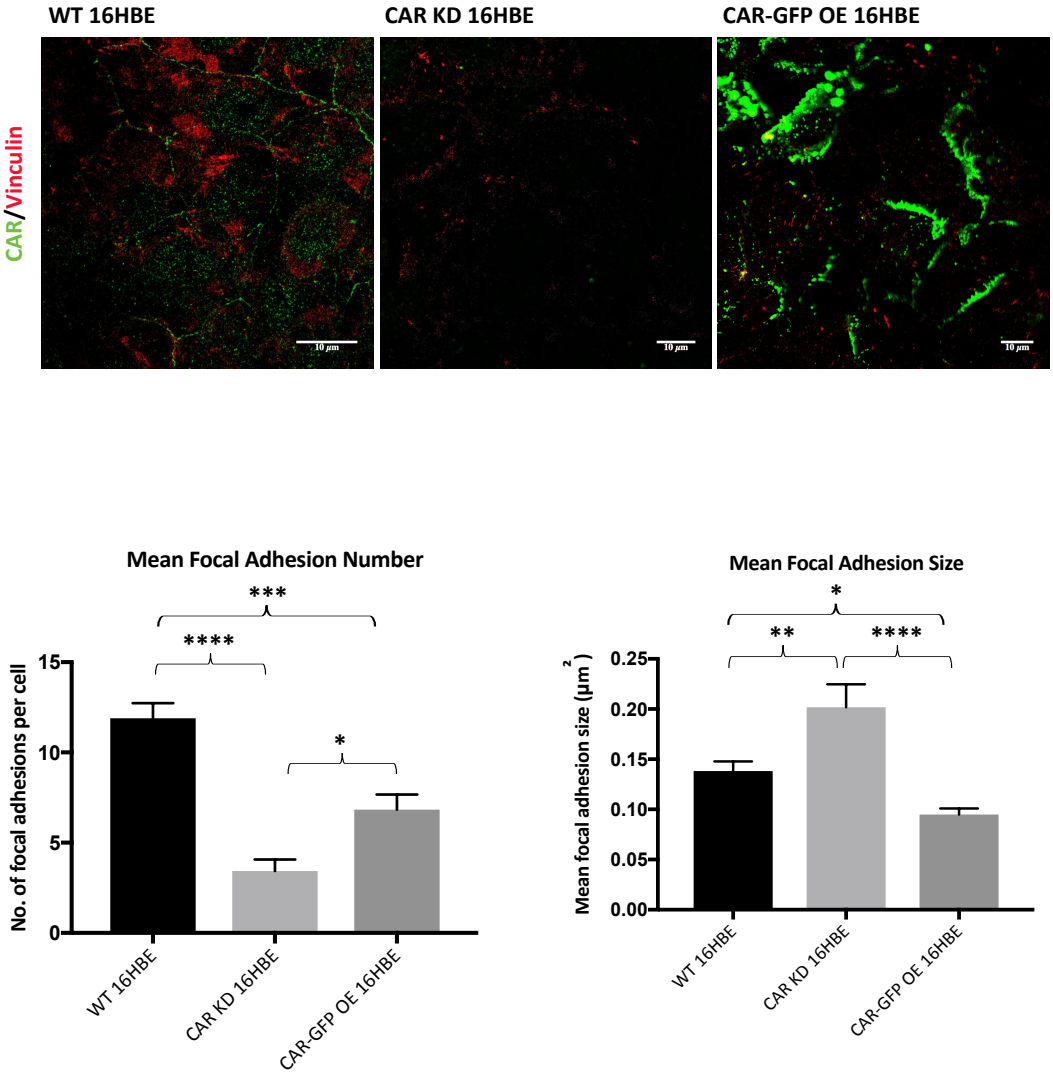
4.3 **Overexpression of CAR alters integrin activation in HBEC.** (A) Western blot of lysates from wilt-type (control), stable CARGFP and and its phospho-mutants overexpressing HBECs probed for Total β1 integrin or Hsc70 as loading control. Cells were lysed with 2% SDS buffer inclusive of protease inhibitors and Calyculin A. (B) Confocal images of monolayers of HBECs over-expressing GFP-tagged FL-CAR and its cyto-tail mutants grown in calcium-containing media and immunostained for total β1 integrin or active β1 integrin (red). (C) Quantification of average scan intensity for monolayers of HBECs immunostained for total or active integrin β1. N= 2 independent experiments, n ≥ 20 cells per cell type. Data are represented as mean±s.e.m. One-Way ANOVA *= p≤0.05, **= p≤0.005, ***= p≤0.0005, ****= p≤0.0001. Scale bar : 10µm.



4.4 **Phosphorylated CAR increases active β1 integrin containing focal adhesion number in HBEC.** (A) Confocal images of monolayers of HBECs grown in calcium-containing media and immunostained for active β1 integrin used to quantify average focal adhesion count normalised to cell numbers in (B) and average focal adhesion size in (C). N= 2 independent experiments, n ≥ 20 cells per cell type. Data are represented as mean±s.e.m. One-Way ANOVA *= p≤0.05, **= p≤0.005, ***= p≤0.0005, ****= p≤0.0001. Scale bar : 10μm.

4.2.3. ***CAR alters focal adhesion number in 16HBE cells***

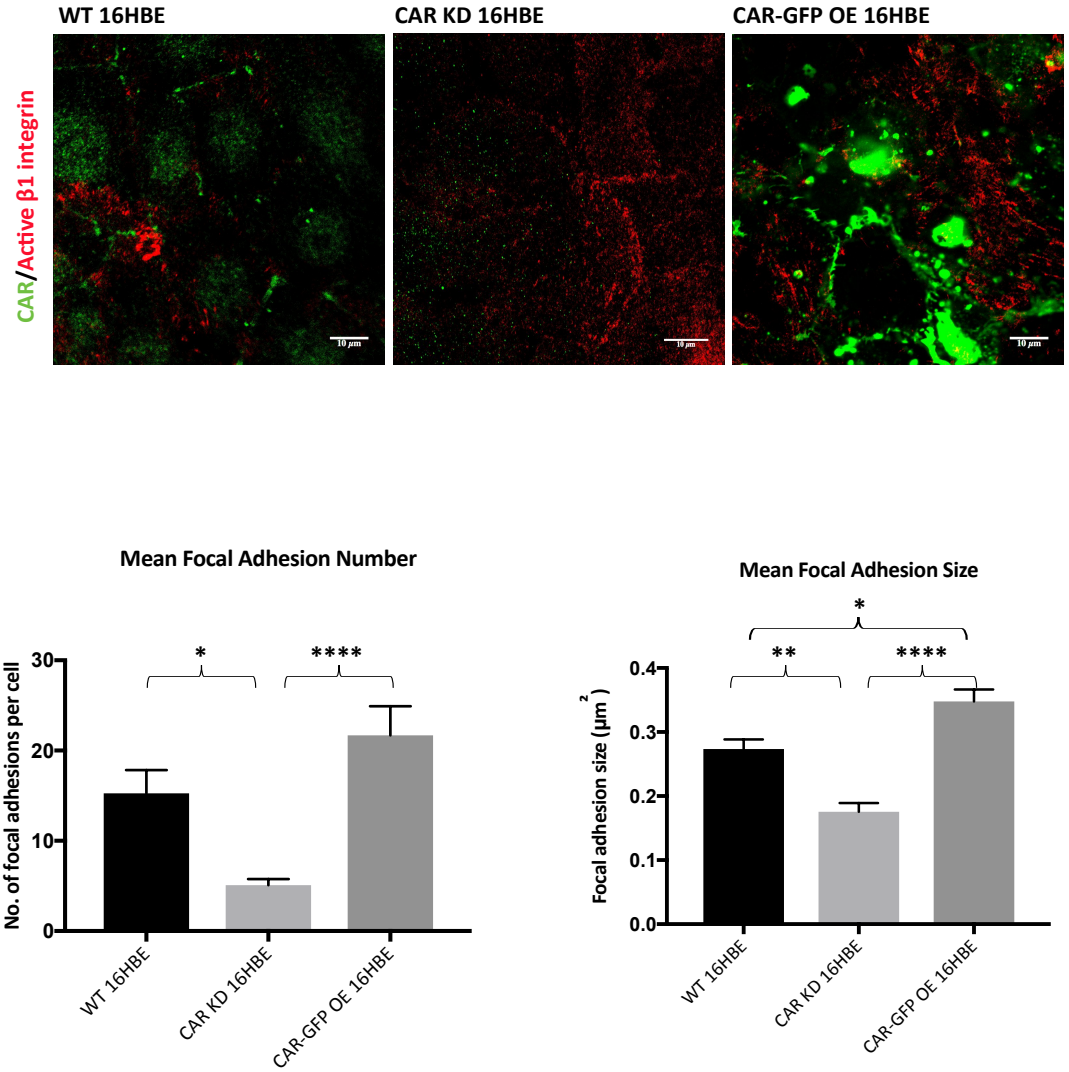
Another human bronchial epithelial cell line 16HBE14o- (hereafter referred to as 16HBE cells), that endogenously express CAR was used to confirm the role of CAR in integrin activation. 16HBE cells were transfected with CAR-specific siRNA (knockdown) or CAR-GFP (overexpression), grown as monolayers in the presence of calcium and immunostained for vinculin. Focal adhesions were then quantified as previously described and in 4.2.1. WT 16HBE had significantly more focal adhesions compared to CAR KD or CAR-GFP over-expressing cells (Fig.4.5). These FA were located beneath or near junctions expressing CAR in both WT and CAR over expressing cells (Fig.4.5A). Analysis of focal adhesion size also showed an increase in CAR KD cells in comparison to WT and CAR-GFP over expressing 16HBE cells. Overall, CAR-GFP over expressing cells had fewer and smaller FA than WT 16HBE. This suggests that CAR can also alter vinculin-containing focal adhesion number in an alternative epithelial cell line.



4.5 **CAR alters focal adhesion number in 16HBE cells.** (A) Confocal images of monolayers of wild-type, CAR knockdown or CAR-GFP overexpressing 16HBE cells immunostained for vinculin (red). Histograms of (B) average focal adhesion count normalised to cell numbers and (C) average focal adhesion size in monolayers of 16HBE cells. N= 1 independent experiments, n ≥ 10 images; ≥ 75 cells per cell type. Data are represented as mean±s.e.m. One-Way ANOVA *= p≤0.05, **= p≤0.005, ***= p≤0.0005, ****= p≤0.0001. Scale bar : 10µm.

4.2.4 .*CAR increases β1 integrin activation 16HBE cells*

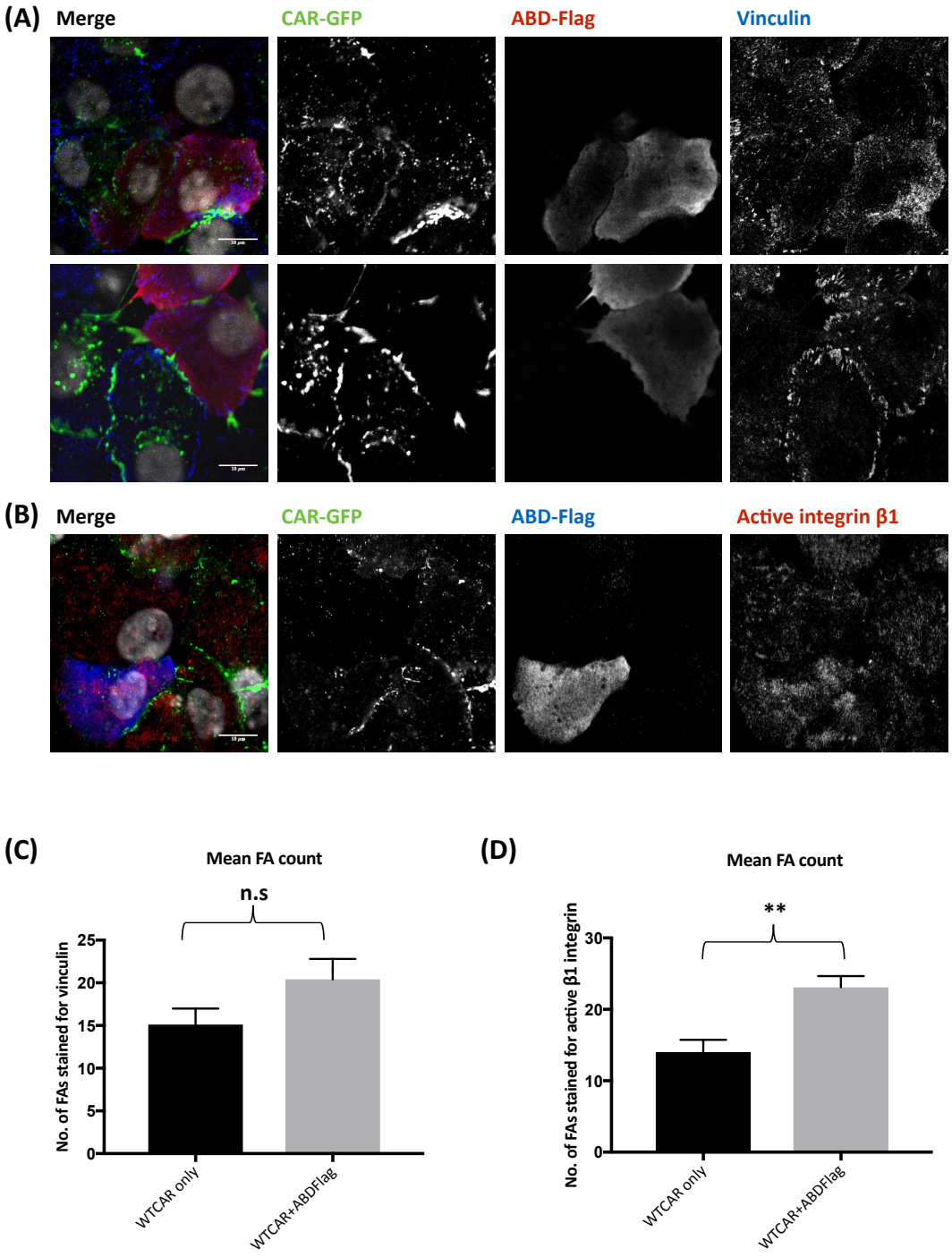
Our lab has previously shown that CAR expression increases integrin activation through increased MAPK activity (Farmer et al 2009). To examine the effect of CAR on active β1 integrin in 16HBE model system, cells were grown as monolayers in the presence of calcium, immunostained for active β1 integrin and quantified for FA size and number as previously described in 4.2.1. CAR KD cells had fewer and smaller FA than WT 16HBE cells, in agreement with data from HBEC. In contrast to the vinculin staining, CAR-GFP over-expressing cells assembled more and larger active β1 integrin containing FA than WT 16HBE (Fig.4.6). This indicates that levels of CAR expression impacts on β1 integrin activation.



4.6 **Overexpressing CAR increases active β1 integrin containing focal adhesion number in 16HBE cells.** (A) Confocal images of monolayers of wild-type, CAR knockdown or CAR-GFP overexpressing 16HBE cells immunostained for active β1 integrin (red). Histograms of (B) average focal adhesion count normalised to cell numbers and (C) average focal adhesion size in monolayers of 16HBE cells. N= 1 independent experiments, n ≥ 10 images; ≥ 75 cells per cell type. Data are represented as mean±s.e.m. One-Way ANOVA *= p≤0.05, **= p≤0.005, ***= p≤0.0005, ****= p≤0.0001. Scale bar : 10μm.

4.2.5. ***Over-expression of FLNa ABD increases FA number in CAR expressing cells***

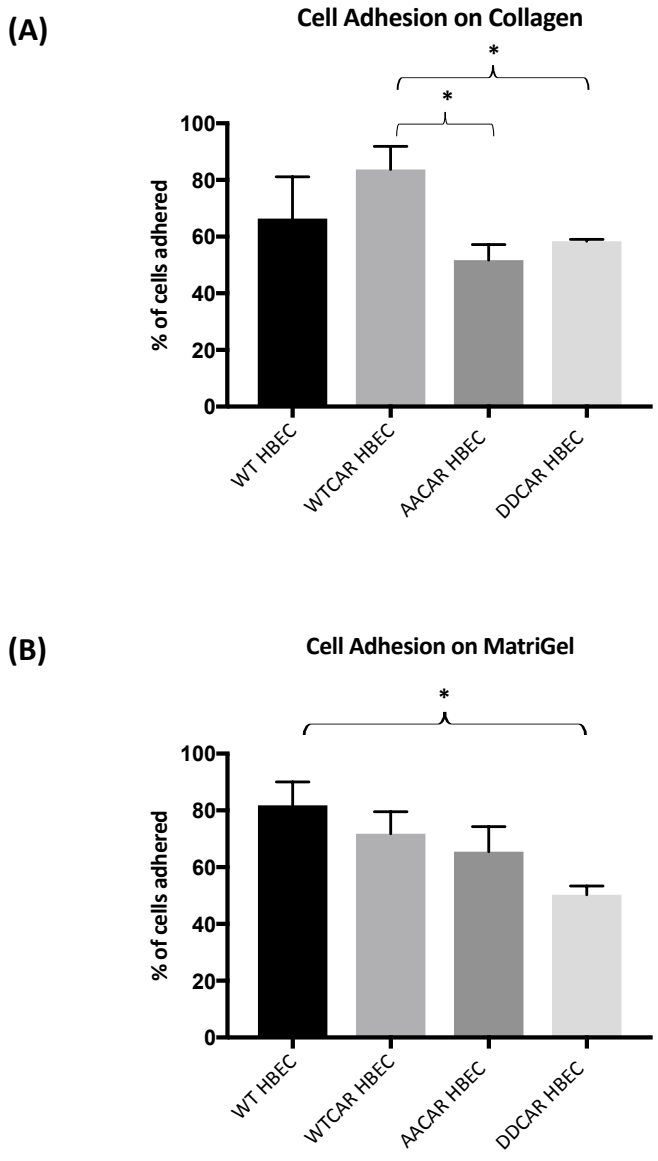
Increased activation of β1 integrin is observed in the presence of phosphorylated CAR. Data from the previous chapter showed that phospho-CAR binds to FLNa, which is a negative regulator of β integrin activation. This suggests that the CAR-FLNa interaction may act as a molecular switch in controlling integrin activation. In the previous chapter, it was shown that phosphorylated CAR binds within the actin-binding domain of FLNa (Fig.3.5 & 3.6). It was also demonstrated that overexpressing CAR increased CAR-FLNa interactions (Fig.3.7). To examine the effect of over-expressing ABD on focal adhesions, WTCAR-GFP HBEC transiently expressing ABD-Flag were grown as monolayers in the presence of calcium and immunostained for flag and vinculin or active β1 integrin. Focal adhesion number was quantified from the confocal images of immunostained cells. Vinculin distribution pattern in WTCAR-GFP/ABD-Flag HBEC were similar to WTCAR HBEC (Fig.4.1) with most of the vinculin containing FA localised to or near CAR positive junctions (Fig. 4.7A). Quantification of number of focal adhesions per cell in cells expressing ABD-Flag showed no significant difference compared to untransfected cells (Fig.4.7C). Active β1 integrin containing FA also localised to CAR positive junctions (Fig.4.7B) and quantification revealed a significant increase in active β1 integrin-containing focal adhesion number in WTCAR HBEC that over-expressed ABD (Fig.4.7D). Thus, the above data indicates that ABD overexpression increases activation of β1 integrin in a CAR-dependent manner.



4.7 **Overexpression of FLNa ABD increases FA number in CAR expressing cells.** (A) Confocal images of WTCAR-GFP HBECs transiently transfected with FLNa-ABDFlag and immunostained for Flag and vinculin or (B) active integrin β1. (C) Histograms of average (C) vinculin-stained or (D) active integrin β1-stained FA count per cell for WTCAR-GFP HBECs overexpressing ABDFlag. N= 2 independent experiments, n ≥ 20 cells per cell type. Data are represented as mean±s.e.m. T-test n.s= not significant, *= p≤0.05, **= p≤0.005, ***= p≤0.0005, ****= p≤0.0001. Scale bar : 10µm.

4.2.6. ***CAR expression alters initial adhesion of HBEC to different ECM proteins***

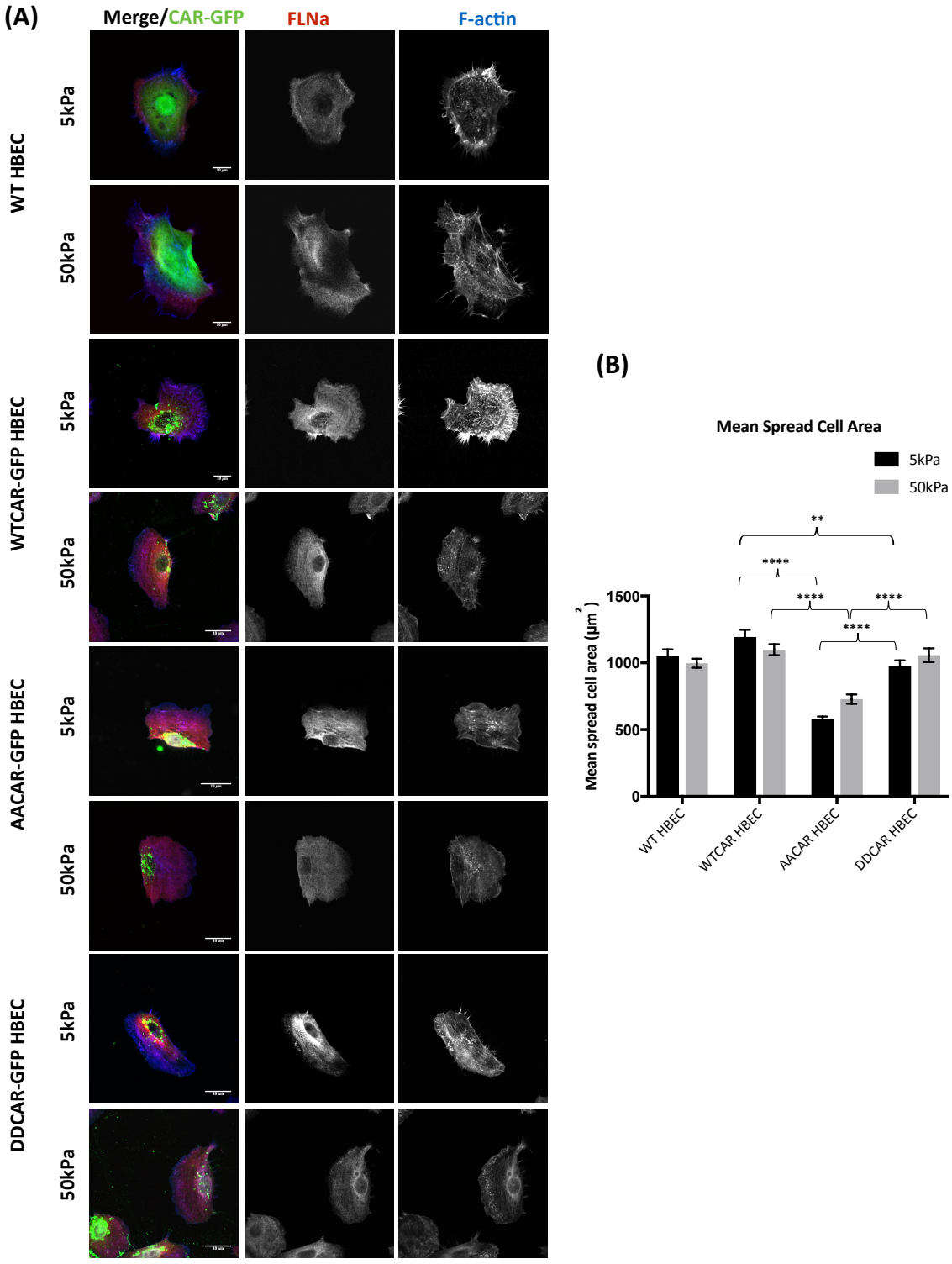
Since phosphorylation status of CAR altered β1 integrin activation, the role of CAR expression in the adhesion of HBEC was examined next. Single WT HBEC, WTCAR, AACAR and DDCAR HBEC were seeded on plated coated with collagen or matrigel, incubated for an hour and fixed. Cells then stained for with Hoechst 33342 to mark the nuclei were quantified as a readout of percentage of cells adhered. No significant difference was observed in the adhesion patterns of the WTCAR cell with respect to control. Both AACAR and DDCAR cells were found to have fewer cells adhered to collagen. On matrigel only DDCAR cells were found to have fewer cells adhered in comparison to WTCAR cells (Fig.4.8A,B). This suggests that CAR phosphorylation status affects the initial adhesion of HBEC to different extracellular matrix proteins.



4.8 **CAR expression does not alter initial adhesion of HBEC to different ECM proteins.** Adhesion assay were performed where known number of WTCAR, AACAR or DDCAR HBECs were seeded on collagen or matrigel coated plates and incubated for 1h before fixation. Cells were stained with nuclear marker, Hoechst 33342 and quantified as percentage of cells adhered to (A) collagen or (B) matrigel. n = 3, data are represented as mean±s.e.m. One-Way ANOVA, *= p≤0.05, **= p≤0.005, ***= p≤0.0005, ****= p≤0.0001.

4.2.7. ***Phosphorylation of CAR regulates spread cell area but not adhesion responses to substrate stiffness***

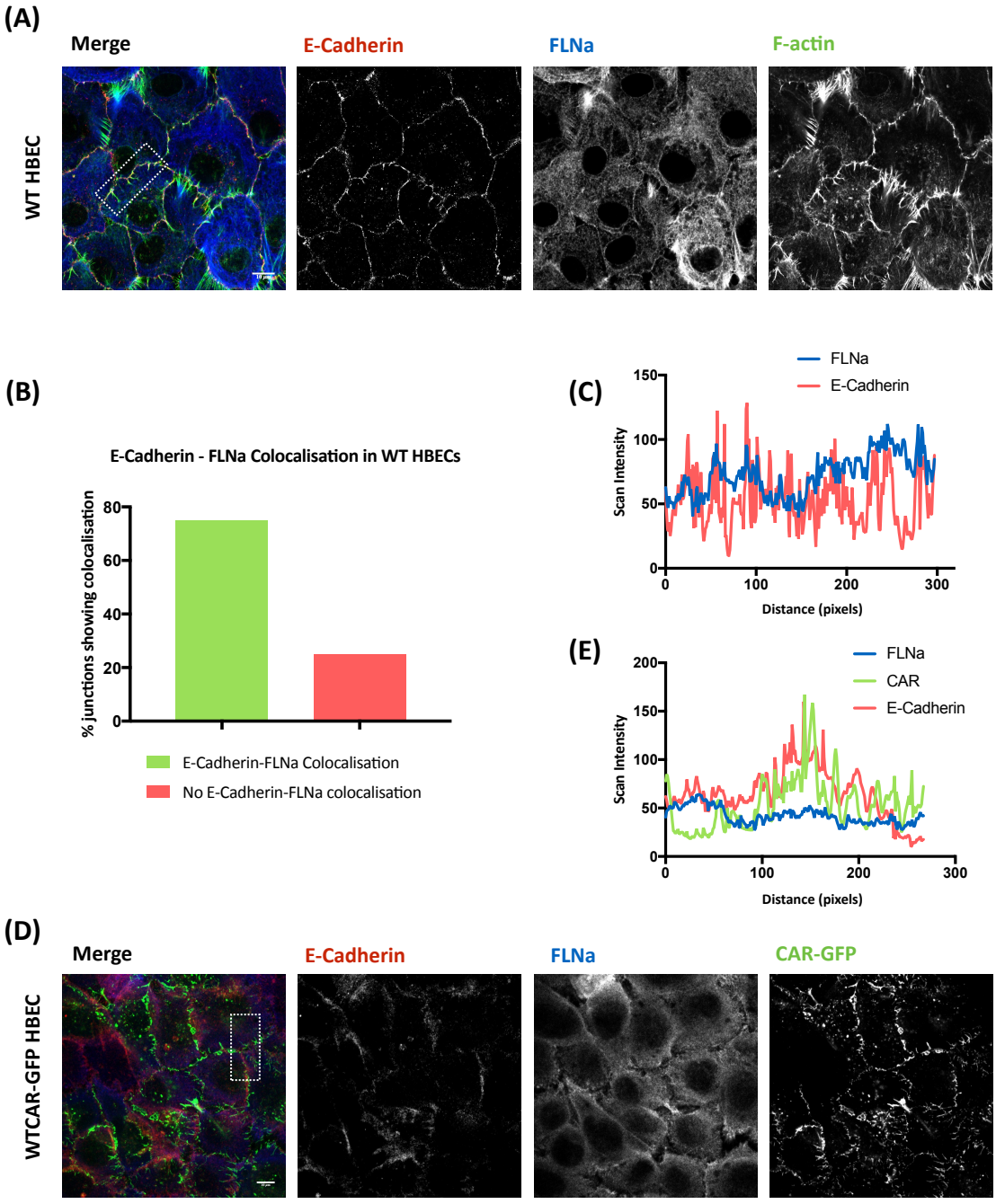
The mechanical properties of extracellular matrix (ECM), particularly its stiffness (or rigidity) as defined by elasticity (or Young's module), can play a role in determining cell adhesion, cell spreading and migration (reviewed in Wells 2008; Mason et al 2012). Given the findings that CAR binds to FLNa, which is a mechanosensitive molecule, and that CAR can also control adhesion size and number, we hypothesised that CAR may also regulate cell-dependent adhesion in response to different substrate stiffness. The effect of rigidity on CAR-dependent cell adhesion was therefore examined by seeding HBEC on substrates of two different stiffness coated with collagen. "Soft" gels with Young modulus of 5kPa and "rigid" gels if 50kPa were made using polydimethylsiloxane (PDMS) blends as described by Palchesko et al (2012). Cell spreading, which involves forming initial contacts with the underlying ECM, was quantified by measuring spread cell area 1h after cells were seeded. Quantification of spread cell area using phalloidin as a marker of peripheral boundaries demonstrated no significant difference in the spread area of the same cell type between the two different stiffnesses. Although WTCAR HBEC did not differ from control HBEC in spread area AACAR and DDCAR HBEC both showed a significant reduction in spread area on 5kPa compared to WT or WTCAR cells (Fig.4.9). AACAR HBEC also showed significantly reduced spread area on 50kPa substrates compared to WTCAR and DDCAR cells. This suggests that CAR phosphorylation may play a role in cell spreading, but not in response to substrate stiffness.



4.9 **Phosphorylation of CAR regulates spread cell area but CAR expression does not alter responses to different substrate stiffness.** (A) Confocal images of WT HBECs or WTCAR, AACAR, DDCAR-GFP HBECs grown on soft (5kPa) or stiff (50kPa) PDMS gels. Cells were immunostained for FLNa (red) and F-actin (blue). (B) Histograms of average cell spread area. N=3, n ≥ 50 cells per cell type. Data are represented as mean±s.e.m. One-Way ANOVA, *= p≤0.05, **= p≤0.005, ***= p≤0.0005, ****= p≤0.0001. Scale bar : 10µm.

4.2.8. ***CAR alters E-Cadherin localisation and interaction with FLNa***

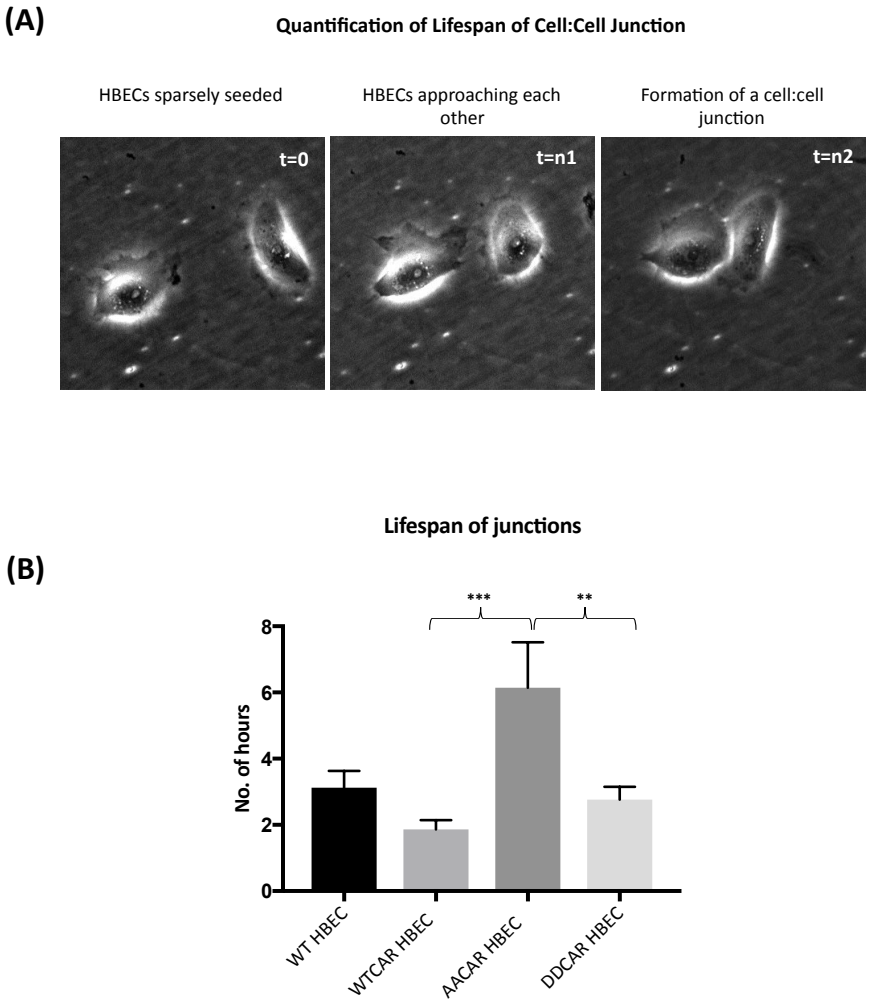
Previous work from our lab has shown that CAR colocalises with E-cadherin at epithelial cell junctions and PKC δ mediated phosphorylation of the CAR cytoplasmic tail controls E-Cadherin localisation at junctions (Morton et al 2013). To examine FLNa recruitment to cell junctions in the absence of CAR, WT HBEC were grown as monolayers in the presence of calcium and immunostained for FLNa and E-Cadherin (Fig.4.10A). Line scans and quantification of cell junction intensity shows colocalisation of FLNa-E-Cadherin at ~75% of cell-cell adhesion sites (Fig.4.10B, C). However, FLNa-E-Cadherin colocalisation reduced in the presence of CAR (Fig.4.10D,E) in agreement with previous studies from our lab showing that overexpression of CAR reduces E-cadherin at cell-cell adhesion sites.



4.10 **CAR alters E-Cadherin localisation and interaction with FLNa.** (A) Confocal images of WT HBEC monolayers grown in calcium-containing media and immunostained for E-Cadherin (red), FLNa (blue) and F-actin (green). (B) Histograms showing the percentage of colocalisation between E-Cadherin and FLNa at cell:cell junctions. N=1 independent experiment, n=40 cell:cell junctions, data are represented as mean. (C) Line intensity scan of E-Cadherin (red) and FLNa (blue) at cell:cell junction in the boxed region in (A). (D) Confocal images of WTCAR-GFP HBEC monolayers grown in calcium-containing media and immunostained for E-Cadherin (red) and FLNa (blue). (E) Line intensity scan of CAR-GFP, E-Cadherin (red) and FLNa (blue) at cell:cell junction in the boxed region in (D). Scale bar : 10µm.

4.2.9. ***CAR phosphorylation regulates cell junction stability***

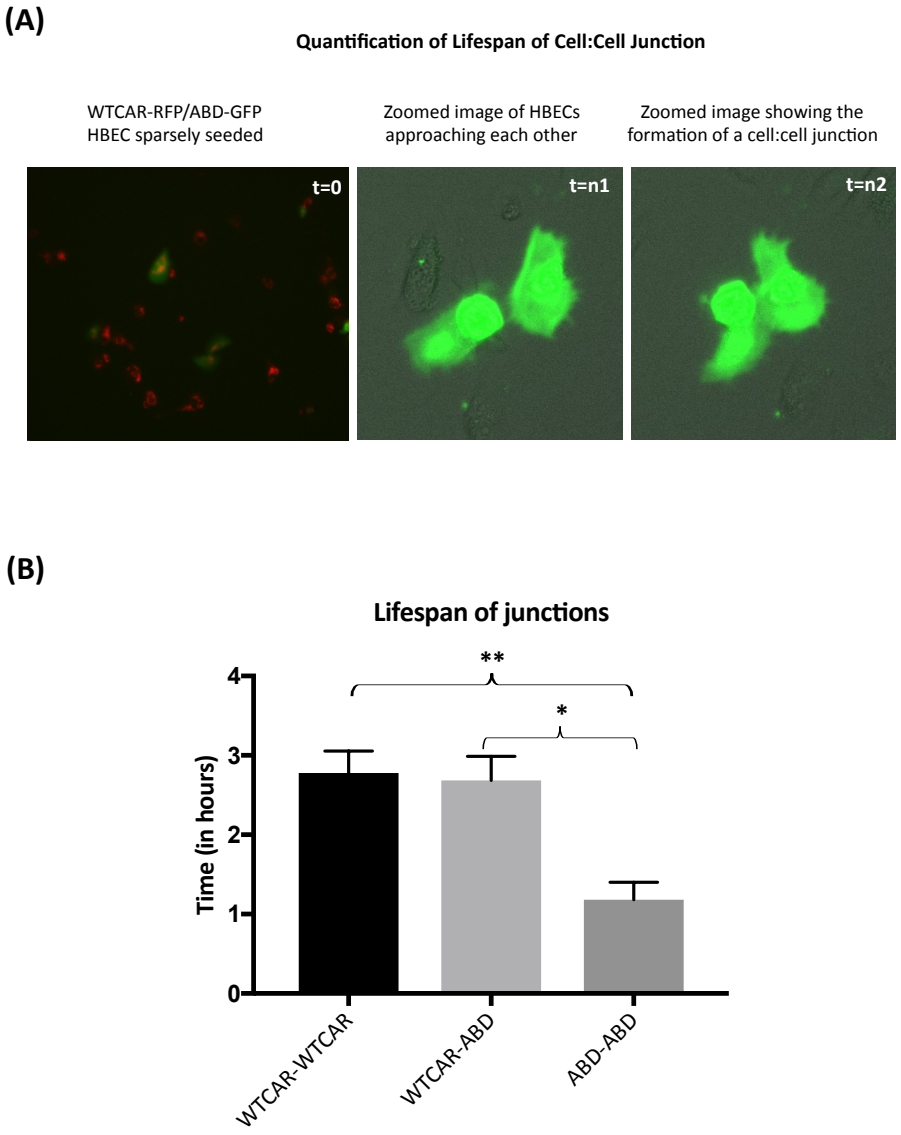
CAR is a cell-cell adhesion molecule and binds across adjacent membranes, however the potential role for CAR in controlling cell-cell adhesion stability remains unknown. To analyse the role of CAR in controlling junction stability, the lifespan of cell:cell junctions formed between freely moving cells were calculated. Single HBEC were seeded sparsely (at 30% confluence) on collagen-coated dishes. Cell movements were monitored using phase contrast/ fluorescent microscope at intervals of 10minutes. Cell junction lifespan was calculated as the duration that cells spend in physical contact to form a junction until they lose physical contact, as shown in Fig.4.11A. WT HBEC on an average showed junctional lifespans of ~3.12 hours, while WTCAR HBEC showed a significant reduction in cell:cell junction duration of ~1.86 hours (Fig.4.11B). Interestingly, AACAR expressing cells showed a three-fold increase in cell-cell adhesion duration (~6.14 hours), whereas DDCAR cells showed a similar junction lifespan to WTCAR cells (Fig.4.11B). Further analysis of the time-lapse movies showed WTCAR and DDCAR cell junctions underwent dynamic junctional association, whereas AACAR cells formed more stable junctions resulting in increased collective cell migration. This data suggests that phosphorylation of CAR regulates cell junction stability.



4.11 **CAR phosphorylation regulates cell junction stability.** (A) Example phase contrast images of sparse HBECs seeded on collagen coated plates in calcium-containing media, at different time points of their movement/ interaction. Lifespan of a cell:cell junction was measured as the average time two interacting cells retain direct physical contact as shown in the last panel in (A). (B) Histograms of average lifespan of cell:cell junctions in WT HBECs, WTCAR, AACAR or DDCAR HBECs. N = 2 independent experiments, n ≥ 10 interacting cell pair per cell type. Data are represented as mean±s.e.m. One-Way ANOVA, *= p≤0.05, **= p≤0.005, ***= p≤0.0005, ****= p≤0.0001.

4.2.10. ***FLN-ABD overexpression alters cell:cell dissociation in a CAR-dependent manner***

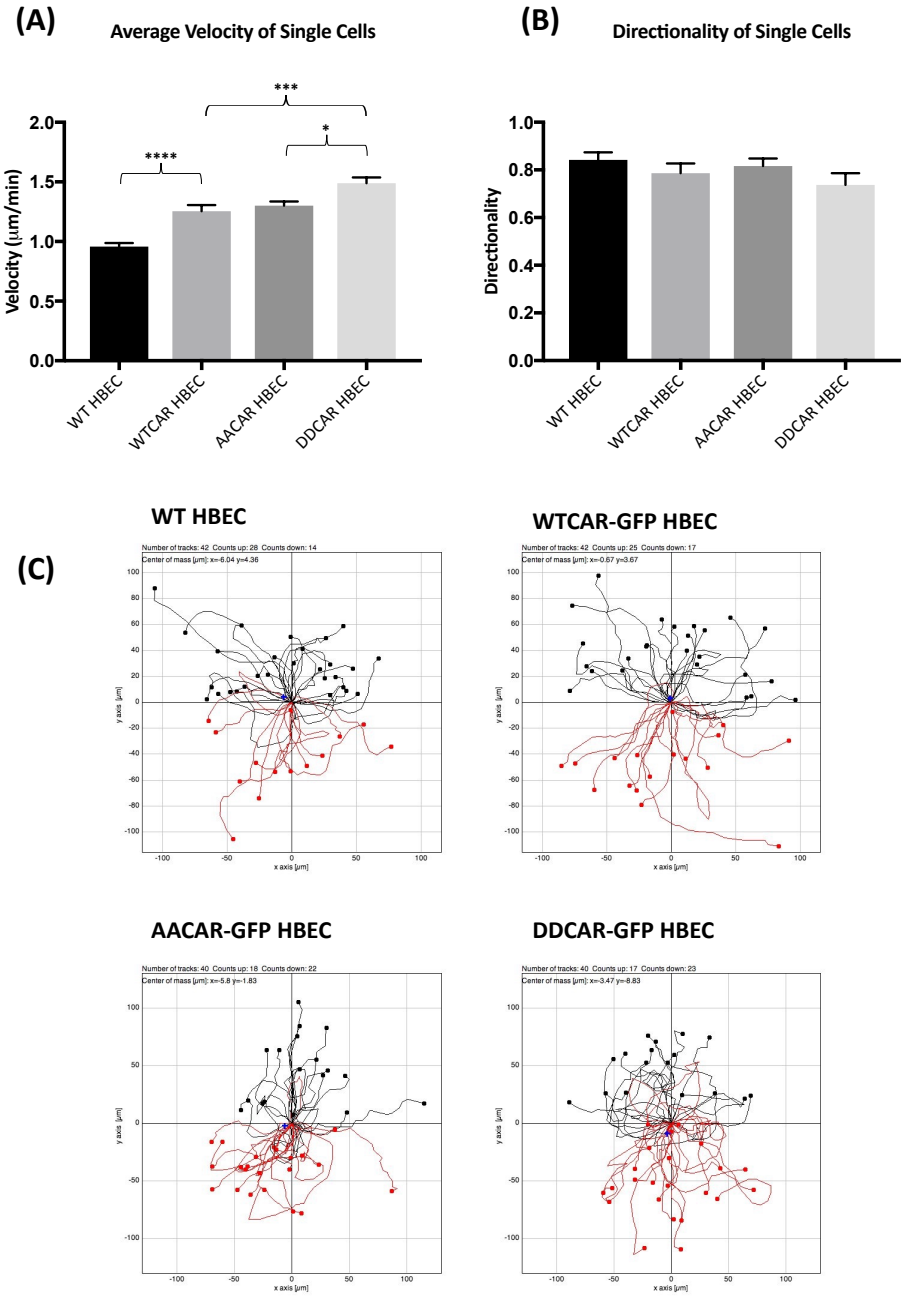
As previous experiments have shown that CAR and FLNa can associate via the ABD of FLNa, the next question was whether the CAR-FLN interaction might participate in the junction instability seen in WTCAR and DDCAR cells compared to AACAR equivalents. To test this, the ABD of FLNa coupled to GFP was transiently transfected into WTCAR-RFP expressing cells and time-lapse experiments performed as in the previous figure (Fig.4.12). The lifespan of cell:cell junctions were quantified between HBEC expressing both WTCAR-RFP and ABD-GFP (referred to as ABD-ABD interaction in Fig.4.12B), two HBEC of which only one expresses both WTCAR-RFP and ABD-GFP (WTCAR-ABD); and cells not expressing ABD-GFP (WTCAR-WTCAR). The lifespan of junctions for WTCAR-WTCAR and WTCAR-ABD interactions were calculated to be 2.77hours and 2.68hours, respectively, while ABD-ABD cell interactions were significantly reduced (1.18hours; Fig.4.12B). This data suggests that over-expressing ABD reduces ability of HBEC to retain cell:cell junctions in a CAR-dependent manner.



4.12 **FLN-ABD overexpression alters cell-cell dissociation in a CAR-dependent manner.** (A) Example fluorescent images of sparse WTCAR-RFP HBECs transfected with ABD-GFP, seeded on collagen coated plates in calcium-containing media, at different time points of their movement/ interaction. Lifespan of a cell:cell junction was measured as the average time two interacting cells retain direct physical contact as shown in the last panel in (A). (B) Histograms of average lifespan of cell:cell junctions in WTCAR-RFP:WTCAR-RFP, WTCAR-RFP:WTCAR-RFP/ABD-GFP expressing HBEC or two WTCAR-RFP/ABD-GFP expressing HBEC. N = 2 independent experiments, n ≥ 10 interacting cell pair per cell type. Data are represented as mean±s.e.m. One-Way ANOVA, *= p≤0.05, **= p≤0.005, ***= p≤0.0005, ****= p≤0.0001.

4.2.11. ***Phosphorylated CAR promotes single cell migration***

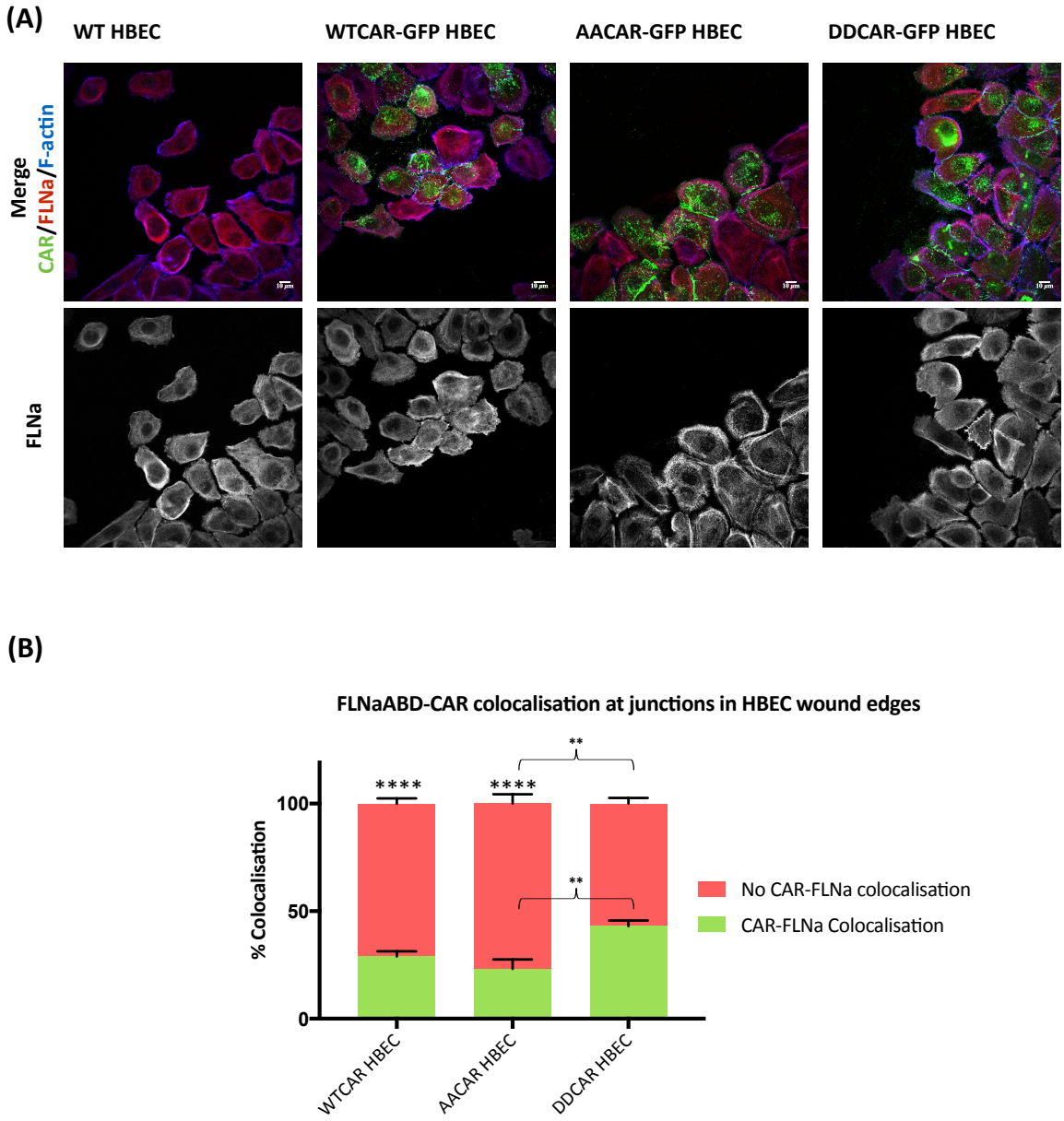
Given that CAR-dependent differences in focal adhesions were seen in both single cells and those in groups, the next questions was whether CAR may play a role in single cell motility. Time-lapse movies were acquired as in the previous figure and cell migration speed and persistence of control, WTCAR and CAR phosphomutant HBEC were calculated by tracking individual cells and processing the resultant tracks using the Chemotaxis and Migration Tool in FIJI. Cell migration speeds (average velocity) were significantly higher in WTCAR, AACAR and DDCAR HBEC compared to WT HBEC controls (Fig. 4.13A,C). Moreover, DDCAR cells showed a significant increase in mean speed above all other cell lines. Directional persistence of migration was unchanged across all four cell types (Fig.4.13B,C). This data suggests that expression of CAR increases migration speed, and that this is more pronounced in cells expressing a phospho-mimic form of CAR.



4.13 **Phosphorylated CAR promotes single cell migration.** Single WT HBECs or WTCAR, AACAR, DDCAR HBECs were grown on collagen coated plates in calcium-free media. Time-course experiment was performed and cells analysed using Cell Tracker on FIJI. Quantification of (A) average velocity or (B) directionality of singles HBECs. N=3, n ≥ 30 cells per cell type. Data are represented as mean±s.e.m. One-Way ANOVA, *= p≤0.05, **= p≤0.005, ***= p≤0.0005, ****= p≤0.0001. Individual cells were tracked and plotted to determine cell persistence as shown in (C).

4.2.12. *CAR and FLNa co-localise during collective epithelial cell migration*

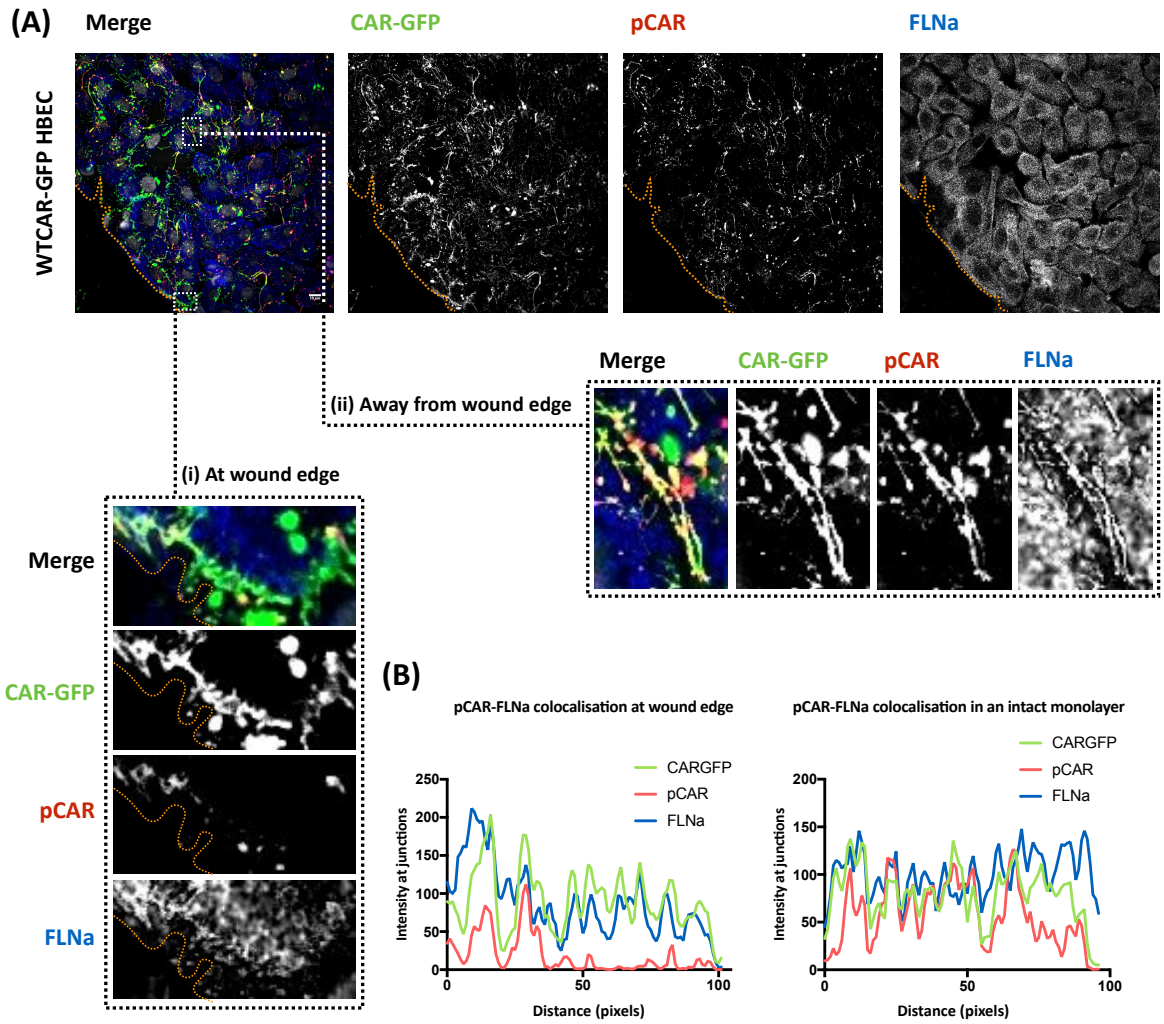
Previous data has indicated that CAR can regulate focal adhesion formation and cell-cell adhesion stability as well as single cell migration. However, as CAR is predominantly localised to cell:cell adhesions in epithelial monolayers, where it co-localises with FLNa, the next question was whether CAR may also play a role in collective cell movement and whether induction of collective migration alters CAR-FLNa association. To test this, HBEC were grown as monolayers in the presence of calcium on collagen coated coverslips and a scratch wound was introduced in the monolayer to promote collective cell migration. Cells were then fixed 2 hours post-wounding, immunostained for FLNa and F-actin and CAR-FLNa colocalisation at cell:cell junctions in cells at the wound edge was quantified. Data demonstrated that FLNa colocalised with all the CAR mutants at cell:cell adhesion sites but to different extents (Fig4.14A). 28.97% of WTCAR expressing cells showed colocalisation with FLNa, similar to AACAR (Fig.4.14B). However, DDCAR showed significantly more CAR-FLNa colocalisation in junctions of cells at the wound edge. This data indicates that CAR and FLNa co-localise at the leading edge during epithelial cell migration and that enhanced CAR phosphorylation promotes this.



4.14 **CAR and FLNa co-localise during epithelial cell migration.** (A) Confocal images at the wound edge of monolayers of HBECs over-expressing GFP-tagged FL-CAR and its cyto-tail mutants grown in calcium-containing media and immunostained for FLNa (red) and F-actin (blue). (B) Histogram showing the percentage colocalisation between CAR and FLNa at cell:cell junctions. N = 3 independent experiments, n ≥ 15 junctions per cell type. Data are represented as mean±s.e.m. One-Way ANOVA, *= p≤0.05, **= p≤0.005, ***= p≤0.0005, ****= p≤0.0001.

4.2.13. ***pCAR levels are lower at the leading edge of collectively migrating epithelial cell monolayers***

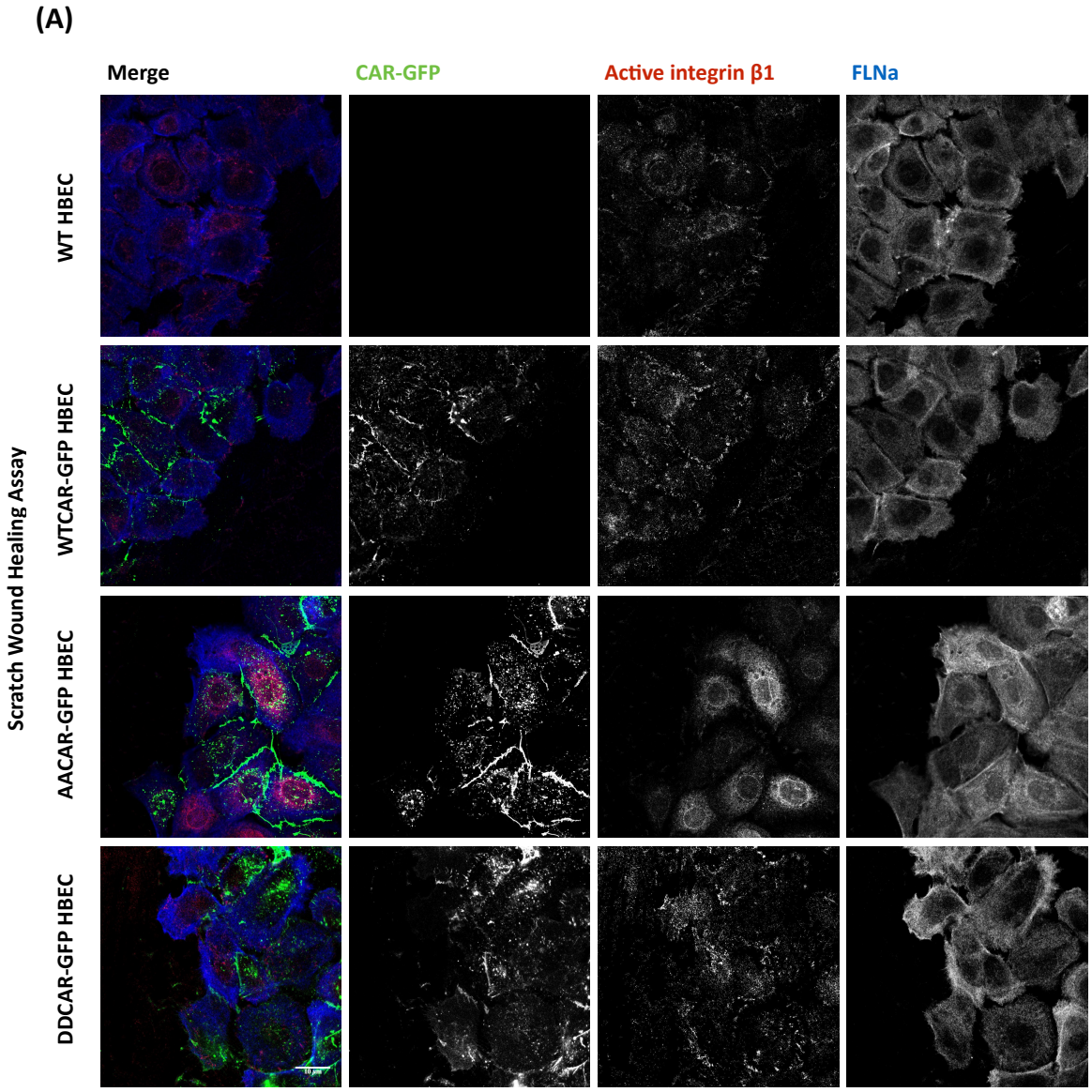
Data in the previous figure showed that a phospho-mimic mutant of CAR enhanced the colocalisation between CAR and FLNa compared to WTCAR, suggesting that levels of endogenous phospho-CAR at the wound edge may be lower compared to cells in a monolayer behind this region. In order to test this, monolayers of WTCAR HBEC were wounded, fixed after 2 hours and immunostained with phosphoCAR and FLNa antibodies. Analysis of resulting images revealed that phosphorylated CAR was seen between cells predominantly in the intact monolayers behind the wound edge (Fig.4.15A). Very little or no phospho-CAR was seen in cells at leading edge of the monolayer as shown in line scans of junctions in Fig.4.15B. This data suggests that endogenous phospho-CAR levels are reduced at leading edge of migrating cells and this may explain the enhanced CAR-FLNa colocalisation in DDCAR cells shown in Fig.4.14.



4.15 **Reduced pCAR-FLNa interaction at leading edge of migrating epithelial cells.** (A) Confocal images at the wound edge of WTCAR-GFP HBEC monolayers grown on collagen coated plates in calcium containing media and immunostained for pCAR (red) and FLNa (blue). Zoomed images of pCAR and FLNa staining shown (i) at wound edge or (ii) away from the wound edge in an intact monolayer of HBEC. (B) Line intensity scan of CAR-GFP, pCAR (red) and FLNa (blue) at cell:cell junctions at wound edge of migration cells or in intact monolayers. Scale bar : 10µm.

4.2.14. ***CAR alters focal adhesion number during epithelial cell migration***

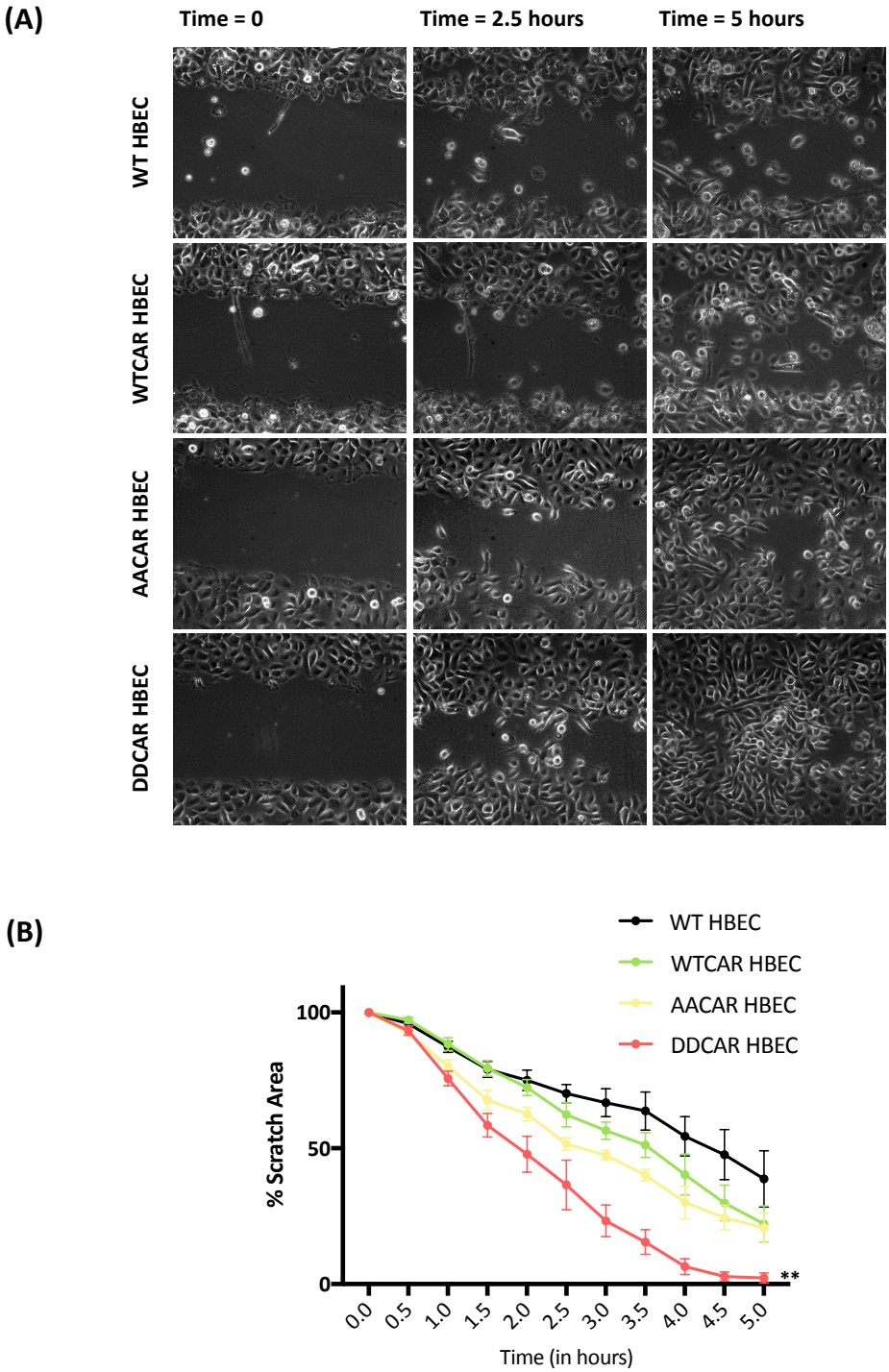
As previous images demonstrated that CAR and FLNa colocalise in junctions of HBEC at the leading edge of collectively migrating cell groups, the next goal was to determine if integrin activation was also different within different populations of cells in a CAR-dependent manner. To this end, WT or CAR-expressing HBEC were immunostained for active β1 integrin following scratch wounding to determine whether any differences in active integrin localisation were present across the monolayers. Images demonstrated that WTCAR and DDCAR HBEC showed prominent focal adhesions as compared WT HBEC (Fig. 4.16); however, the signal for active integrins appeared to be higher in DDCAR cells at the leading edge compared to WTCAR cells. In contrast, AACAR HBEC showed fewer and smaller adhesions containing active integrin both at the leading edge and behind within the monolayer. This data suggests that CAR regulates integrin activation at the leading edge during epithelial cell migration.



4.16 **CAR alters focal adhesion number during epithelial cell migration.** (A) Confocal images at the wound edge of monolayers of HBECs over-expressing GFP-tagged FL-CAR and its cyto-tail mutants grown in calcium-containing media and immunostained for active β1 integrin (red) and FLNa (blue). Scale bar : 10μm.

4.2.15. ***Constitutive CAR phosphorylation accelerates collective cell migration***

As previous data showed that phosphorylated CAR associates with FLNa, and that DDCAR showed increased FLNa binding at the leading edge of cells in groups, coupled with potentially increased active integrin, the next goal was to determine whether expression of CAR or mutants forms of therein may regulate collective migration of epithelial cell monolayers. WT HBEC or those expressing WT or mutant CAR were analysed by time-lapse microscopy following wounding and the ability of cells to close the wound over time was quantified. Speed of collective epithelial cell migration was calculated as the percentage in reduction of scratch area over time. Representative images of example stills from movies of each cell line are shown in Fig 4.17A. WTCAR and control HBEC demonstrated similar wound closure rates over the first 2 hours, but WTCAR HBEC then showed increased wound closure by 5hours (Fig 4.17B; black and green lines). AACAR expressing cells showed faster wound closure rates than WT and WTCAR cells, but the resulting wound closure level was the same as WTCAR cells by 5h (Fig 4.17B, yellow line). However, DDCAR HBEC showed accelerated wound closure rates from 1.5h onwards and had fully closed the wound by 5h, significantly faster than both WTCAR and AACAR cells at the same time point (Fig 4.17B, red line). This data suggests that CAR phosphorylation accelerates collective cell migration.



4.17 **Constitutive CAR phosphorylation accelerates collective cell migration.** (A) Phase contrast images of scratch wound healing assay showing the migration of WT HBECs or WTCAR, AACAR or DDCAR HBECs towards the the wound at time 0, 2.5hours or 5 hours. Monolayers of HBECs were grown and scratched 1hour before performing a time course experiments to monitor the closure of the wound introduced by the scratch. (B) Cell migration speed was quantified as a measure of wound closure (i.e, the percentage of scratch area). N=3, n = 5 fields tracked per cell type. Data are represented as mean±s.e.m. Two-Way ANOVA, Tukey's, *= p≤0.05, **= p≤0.005, ***= p≤0.0005, ****= p≤0.0001.

4.3 **Discussion**

FLNa is a key protein in regulating cell membrane stability and cell:matrix signalling pathways (Stossel et al 2001; Bellanger et al 2000; Vadlamudi et al 2002) and FLNa- β1 integrin binding is important for adhesion dependent cell survival (Kim et al 2008). FLNa- β1-integrin is also known to tune epithelial cells in response to changes in ECM composition and thus act as a bidirectional mechanosensitive complex (Gehler et al 2009). This makes CAR, through its interaction with FLNa, a potential candidate in regulating this complex. Data in this chapter has revealed a novel role for CAR in controlling epithelial cell behaviour and migration through the regulation of focal adhesion protein, β1 integrin.

4.3.1. ***CAR has different effects on cell adhesion and migration in single epithelial cells versus monolayers***

CAR expression reduces the ability of phosphorylated CAR to adhere to ECM proteins indicating it has an impact on integrin activation. The reduction may be attributed to the drastically reduced interaction of CAR with FLNa in single cells. However, the absence of effect on early adhesion to ECM in WTCAR and non-phosphorylated mutant shows that cells are able to constitutively activate integrins irrespective of CAR phosphorylation status. In addition, non-phosphorylated CAR has consistently reduced focal adhesion number and size per cell. This indicates the lack of any effect on the integrin binding capacity of FLNa when CAR is non-phosphorylated. The expression of phosphoCAR may instead promote integrin activation after adhesion through FLNa. This suggests that CAR-FLNa binding could be constitutive and may not

necessarily require CAR to be homodimers at cell:cell junctions. Altogether, CAR appears to have an effect on the initial cell adhesion to ECM proteins but not necessarily through direct filamin binding.

Filamin binds β1 integrin to stabilise it at resting phase. The knockdown of filamin increases in β1 integrin activation and thereby focal adhesion assembly (reviewed in Calderwood 2004; Kiema et al 2006). This is consistent with data from single phosphoCAR cells, which show an increase in focal adhesions and cell speed during migration. This accompanied by the reduced initial cell adhesion suggests a constitutive inactivation of FLNa with respect to integrin binding in phosphoCAR cells. It is also worth noting that WTCAR, which is not constitutively phosphorylated in single cells, has fewer focal adhesions but increased cell migratory speed. This is likely due to signalling to ERK, which has been implicated in controlling epithelial cell migration (Sharma et al 2003; Matsubayashi et al 2004).

In epithelial monolayers, WTCAR and its phosphomutants localise at cell:cell junctions and exhibit a more pronounced effect on epithelial cell behaviour and function. PhosphoCAR promotes colocalisation of FLNa to cell junctions and enhances focal adhesion number and β1 integrin activation. This is consistent with the idea that phosphoCAR binds with greater affinity to FLNa and, therefore, reduces FLNa binding to β1 integrin promoting the latter's activation.

4.3.2. ***CAR regulates duration of cell:cell adhesion***

In single cells beginning to form cell:cell contacts, non-phosphorylated CAR leads to more stable junctions being formed that are maintained for longer durations. As single cell migration speeds of non-phosphorylated CAR are the same as WTCAR cells, this means phosphoCAR becomes important only in single cells when they collide and form a junction. In this situation, the CAR is engaged across junctions and this appears to be stabilised when CAR is constitutively dephosphorylated. This shows phoshoCAR might destabilise the CAR:CAR homodimers and that CAR may become phosphorylated again as the need arises but only after forming a stable homodimer across a junction. This suggests that the CAR-FLNa interaction is not required for initial CAR:CAR stabilisation at a junction, as AACAR does not bind FLNa.

E-Cadherin coordinates the organisation of cell junction proteins through PKCδ in a Ca^{2+} -induced manner (Lewis et al 1994). This perhaps is the mechanism through which CAR is recruited to junction to stable CAR:CAR homodimers in the presence of Ca^{2+} despite the fact that CAR itself does not require the presence of calcium ions to homodimerise. Analysis of CAR distribution at cell:cell junctions in the cells knocked down for PKCδ and in cells treated with inhibitory anti-E-cadherin antibody in the presence and absence of PKCδ activators such as phorbol-12,13-dibutyrate (PDBu) will reveal the role of E-cadherin and PKCδ in recruitment and stable assembly of CAR at junctions.

Interestingly, the expression of Actin Binding Domain (ABD) of FLNa reduces junction lifespan. However, overexpressing ABD promotes CAR-FLNa binding. ABD may promote phosphorylation of CAR through the recruitment or regulation of PKCδ, which localises at cell:cell junctions in an F-actin dependent manner in airway epithelial cells (Smallwood et al 2005; Chen et al 2016). Perhaps the close proximity of both ABD and PKCδ to F-actin might prompt the former to regulate PKCδ activity. GST pulldown of PKCδ in the presence and absence of ABD in epithelial cells can reveal the possibility of this activation mechanism. Further, screening for CAR-FLNa localisation and coimmnoprecipitation in PKCδ knockdown epithelial cells overexpressing ABD might shed some light on this assumption. Overall, these data suggest CAR-FLNa binding and phosphorylation of CAR may destabilise junctions and promote migration.

4.3.3. ***CAR phosphorylation is spatially regulated in monolayers and promotes collective cell migration***

CAR colocalises with FLN at cell:cell adhesions and this interaction is enhanced in phosphoCAR cells. However, CAR has only been found to phosphorylate at cell:cell adhesions in intact monolayers, not on single cells or at the leading edge of collectively migrating cells. Intact monolayers of WTCAR cells have fewer but larger adhesions compared to wild type HBEC. Both phosphomutant lines have reduced focal adhesion size but phosphoCAR cells have more focal adhesions, which correlates with its higher migration speed in the wound assay. The phosphoCAR cells also show higher CAR-FLNa binding at rear end of the cells at the wound edges, where

phosphoCAR is enriched. This might promote increase in integrin activation and adhesion dynamics, which drive wound closure. However, the fact that CAR is not phosphorylated at the front end of cells at wound edges, suggests either that PKCδ is activated only in intact monolayers and not in response to wounds. This could be the mechanism by which epithelial cells promote more stable junctions by expressing non-phosphorylated CAR (refer to section 4.3.2 to see how this mutant promotes junction stability) between rapidly moving cells at the front of a wound.

β1 integrins crucial in wound closure in a number of different types of epithelial cells (Grose et al 2002; Larjava et al 2011; Yamaguchi et al 2015). They are known to drive cell migration at wound edge via Rac activation (Yamaguchi et al 2015). They also induce PKCδ to promote cell junction formation (Ozaki et al 2007). This maybe another reason for the differential activation of PKCδ. The spatial regulation of phosphoCAR, to maintain the precise control of β1 integrins at wound edge may hence contributes to both cell:cell and cell:matrix adhesion stability through the regulation of FLNa function by CAR.

In conclusion, this chapter demonstrates how CAR facilitates “adhesive crosstalk” between cell:cell and cell:matrix adhesion sites, something that is currently very poorly understood. It also reveals the importance of post-translational modification of CAR cytotail, which regulates integrin activation, thereby epithelial cell migration and cell junction stability based on its phosphorylation status. The selective phosphorylation of CAR at the time of

cell:cell contact formation and in recruiting FLNa to cell: cell junction, promotes migration unless there is a cue to switch off the FLNa binding in stable epithelial monolayers.

5. Final Discussion

Despite CAR being first described as a viral docking receptor in 1997, the importance of CAR as a junction protein has only been relatively recently appreciated. Moreover, there is still little known about the regulation of CAR as a junction protein, its recruitment to cell junctions, its binding partners, the molecular mechanism and biological significance behind these interactions.

Work presented in this thesis demonstrates that Filamin A is a novel binding partner of CAR. It potentially associates with CAR through its Actin Binding Domain (ABD) and Rod2 domain, in an F-actin dependent manner. The CAR-FLNa binding is significantly higher when CAR cytoplasmic tail is phosphorylated at threonine290 and serine293 residues. This binding is also higher in cells grown as monolayers in the presence of calcium. Coincidentally, phosphorylated CAR levels are higher under these conditions. It, therefore, indicates that FLNa preferentially associates with CAR when the latter homodimerises *in trans* at cell:cell junctions. CAR also promotes cell migration and maintains junction stability between epithelial cells. While phosphorylated CAR may be important to assemble a junction, it is the non-phosphorylated CAR that plays a role stabilising it. A summary of the key findings from this thesis is depicted in Figure 5.1. The broader potential impact of these findings are discussed below.

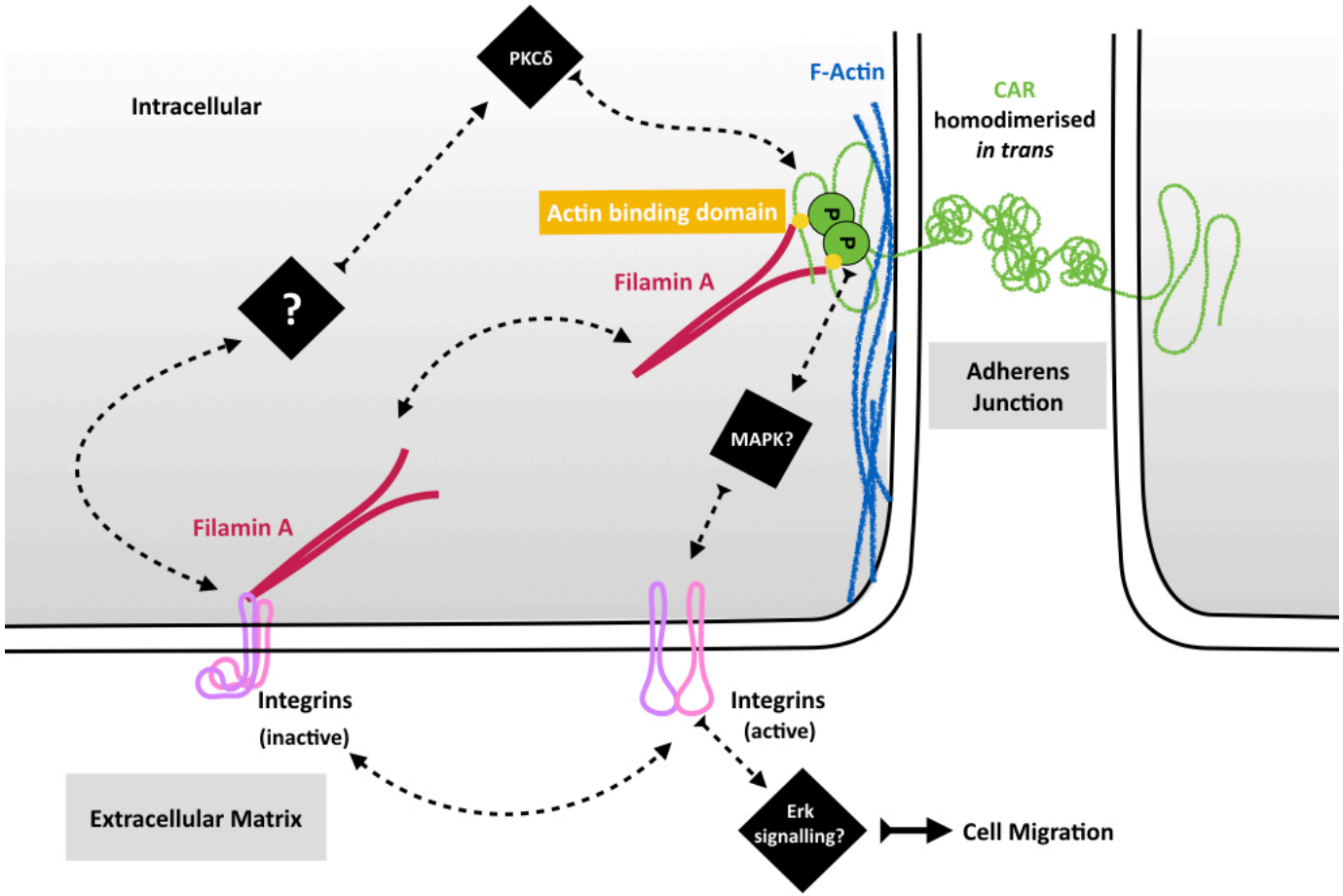


Fig.5.1. **Proposed model for the interaction between CAR and FLNa.** (1) CAR cytotail phosphorylated by PKCδ, (2) FLNa displaced to cell:cell junctions to bind pCAR through its Actin Binding Domain, (3) Integrin activated via MAPK activity induced by pCAR.

5.1. ***CAR homodimerisation promotes CAR phosphorylation and FLNa binding***

Transmembrane protein dimerisation and subsequent assembly of cell:cell junctions have the potential to change the conformation of the receptors and play an active role in altering the local chemistry and cell architecture (Dawson et al 2005; reviewed in Ratheesh, &Yap 2012 and Zaidel-Bar 2013). During the assembly of cell:cell contacts, cadherin-based junctions mediate the formation of two spatially distinct actin populations: the junctional actin bundles and peripheral thin actin filaments. Over time, they interchange positions and emerge into cortical actin rings that confer stability and polarity to epithelial cells (Zhang et al 2005). My data revealed reduced FLNa localisation at wild-type CAR expressing junctions and both CAR and FLNa at junctions in phosphomemetic mutants upon disruption of F-actin, suggesting a role for F-actin in reinforcing CAR-FLNa complex stability at junctions. In fact, the formation of CAR-FLNa complex itself may be dependent on F-actin acting as a scaffold to orchestrate their binding. Alternatively, FLNa may need to be in an actin bound state to assume a conformation conducive to associate with CAR. These assumptions can be substantiated by my observation of the high affinity binding of CAR to the ABD of FLNa and previous study showing the association of actin with CAR (Huang et al 2007). It is also worth noting that a stable cortical actin ring is more likely to facilitate CAR-FLNa binding. This is evident from the fact that CAR binds FLNa readily at cell junctions but very little or no binding is found in single epithelial cells that mainly comprise loose actin meshwork at its lamellipodia or stress fibres that are rapidly turned over in response to migratory cues (reviewed in Tojkander et al 2012; Lehtimäki et al 2016).

My data also reveals that CAR-based junctions are not static. Epithelial cells in intact monolayers shuffle around each other and cell:cell contacts must, therefore, be plastic in nature to accommodate this repositioning. From the cell:cell junction lifespan data (Fig.4.11), it is evident that phosphomemetic CAR expressing cells exhibit a more dynamic junction movement phenotype whereas the non-phosphorylated mutant show more stable junctions. This evidence leads to the argument that CAR is transiently phosphorylated in response to external stimuli such as inflammatory cytokines (Morton et al 2016) or F-actin dependent changes as described above, to facilitate FLNa binding, which in turn promotes junction remodelling. This binding increases the speed of collective migration in the epithelial population as shown in the wound-healing assay (Fig.4.17). Together, these findings suggest a role for CAR-FLNa binding in growth and differentiation of tissues, and more importantly in repair mechanism following an injury. Tracking CAR-FLNa colocalisation in leader and follower cells of a migrating epithelial population under homeostatic and inflammatory conditions and analysing migratory speed *in vivo* in CAR knockout murine model system will provide a better understanding of the interplay between the two proteins.

Another striking feature of the binding between phosphorylated CAR and FLNa is its enriched localisation at tricellular junctions. These are sites of high tension at cell membranes between three or more epithelial cells (Bosveld et al 2016) and are characterised by the presence of spiky actin stress fibres and short thick filamin bundles. E-Cadherin has previously been suggested to play a role in supporting these structures (Borghi et al 2012). It is feasible that CAR may also be an important regulator of tricellular junction maintenance through interactions with FLNa and F-

actin. More detailed analysis of these specific tricellular adhesions would be required in future to dissect this further.

5.2. ***Biological significance of CAR-FLNa interaction and its regulation***

Previous studies from our lab have shown that CAR is phosphorylated on threonine290/serine293 within the cytoplasmic tail by the serine-threonine kinase PKCδ (Morton et al 2013). Data shown in this thesis demonstrates that one or both of these sites need to be phosphorylated in order for CAR to bind FLNa. Cells expressing the phosphomimetic mutant of CAR showed a dramatic increase in both focal adhesion number and migratory speed. This is likely due to two events happening simultaneously: (i). Filamin A is displaced from binding to β1 integrins, thereby allowing the latter to bind activators such as talin and promote focal adhesion assembly (Loo et al 1998; Liu et al 2015); (ii). Phospho-CAR may be indirectly inducing activation of integrins via the p44/42 MAP kinase pathway as our lab have shown previously in MCF7 human breast carcinoma cells (Farmer et al 2009).

FLNa binds directly to the cytoplasmic tail of β1 integrins through its Ig repeats 21-24 (Loo et al 1998). FLNa is thought to facilitate inside-out signalling of integrin β1 by promoting integrin-ligand binding in epithelial cells, which is required for cell spreading and survival in response to external forces (Gehler et al 2009; reviewed in Kim, & McCulloch 2011). However, increased FLNa binding to β1 integrins also promotes integrin inactivation resulting in reduced cell polarity and transient membrane protrusion thereby reducing cell migration (Kim et al 2008; Calderwood et al 2001). It is plausible therefore that data in this thesis showing enhanced

migration in HBEC overexpressing CAR may be due to reduced FLNa- β1 integrin association. The increase in migratory speed was more pronounced during collective cell migration in the wound-healing assay. As data presented in this thesis showed that homodimerisation of CAR is a prerequisite to bind FLNa, the CAR-dependent formation of cell:cell adhesions and coupling to the actin cytoskeleton may further assist in this re-direction of FLNa to junctions to enable integrin activation (reviewed in Ilina, & Friedl 2009). Indeed, cells expressing the phospho-mimetic form of CAR, which binds FLNa with high affinity than WT CAR, migrate faster collectively, whereas the phospho-dead mutant cells migrate slower. This is in potential contrast to other data shown in this thesis that cell:cell adhesions that are stronger and less transient in the AACAR cells compared to DDCAR or WTCAR expressing cells. This suggests that cell:cell adhesion strength and the potential transmission of mechanical forces through these junctional complexes requires a cycle of phosphorylated CAR to be present to ensure recruitment of FLNa and co-ordinated subsequent integrin activation.

It seems likely that CAR and FLNa binding is spatially and temporally regulated in epithelial cells but the precise mechanisms that co-ordinate this remain unclear. However, some clues about potential future aspects for analysis have been provided by observations in this thesis. Firstly, a consistent finding was the presence of higher levels of pCAR and FLNa co-localisation at tricellular junctions (described in section 5.2; Fig.6.1). Another interesting observation from this thesis is increased initial spreading of epithelial cells expressing DDCAR expressing grown on collagen coated both on coverslips or elastic matrix. FLNa regulates β1 integrin activation status to support cell adhesion and cell spreading (Kim et al 2008; Gehler et al 2009) and

whilst very little or no interaction and no colocalisation of CAR-FLNa is observed in single cells (Fig.3.14). It is possible that expressed DDCAR acts to artificially recruit and promote this complex formation, despite the lack of CAR homodimerisation *in trans.* In this situation, DDCAR may be sequestering FLNa away from integrin/ adhesion sites even in single cells and promoting activation of integrins.

The majority of documented interactions of CAR with other proteins in all cell types (refer to Table 1.2) occur through its unstructured cytoplasmic domain. Disordered proteins, which lack well-structured three-dimensional fold, have been implicated in undertaking multiple interactions with varying consequences dictated by post-translational modifications (Wright, & Dyson 2014). Early reports suggested that a stable 3D structure of canonical domains with proteins is required for stable association with binding partners. However, unstructured regions of proteins, including CAR, also have highly conserved amino acid sequences between species and important roles to play in cell signalling (reviewed in Wright, & Dyson 2005). The unstructured nature of the CAR cytoplasmic domain makes it unlikely that any conformational changes in the extracellular domain of CAR (such as might be expected to occur during homodimersation) would impact on the conformation of the cytotail. It is likely, however, that CAR may also cluster to form oligomers *in cis* and this may in turn impact on local packing of the cytoplasmic tail at the plasma membrane leading to changes in availability of certain binding sites for cytoplasmic adaptor proteins. Post-translational modification of the cytotail would also be predicted to alter this organisation of receptor clusters, potentially impacting on function. It would be interesting to use approaches such as super-resolution

microscopy in future to better resolve these potential differences in CAR organisation at the nanoscale at the plasma membrane of epithelial cells.

5.3. ***Role of CAR-FLNa binding in disease***

A recent study has revealed an important role for filamin A in epithelial defence against cancer (EDAC). FLNa accumulates in the healthy epithelial cells at the interface with transformed cells in a Rho/Rho kinase-dependent manner. It regulates dynamic movement of vimentin in normal cells to prevent the formation basal extension by the transformed cells and, therefore, forcing it to be apically extruded (Kajita et al 2014). CAR was found upregulated at cell:cell junctions in lung cancer and is associated with poor survival rates (Wang et al 2006; Wunder et al 2013; Ortiz-Zapater et al 2017). CAR has, however, not been studied in the context of extrusion of transformed cells from a healthy epithelium. My study has shown that affinity of FLNa for phosphorylated CAR and the dynamic regulation of cell junctions (section 5.2). This suggests CAR may have a role to play in EDAC by assembling cell:cell contacts between healthy and transformed cells to aid FLNa accumulation.

FLNa is upregulated during TGF-β induced epithelial to mesenchymal transition (EMT) of A549 lung cancer cells, although its functional role is not well understood in this context (Keshamouni et al 2006). A recent study in colon adenocarcinomas has shown that silencing FLNa reduces focal adhesion size and promotes migration in invasive cells, contrary to its inherent function of inhibiting integrin (Wieczorek et al 2017). A study by Behrens et al., showed that tyrosine phosphorylation of E-cadherin/ β-catenin complex causes invasiveness and dedifferentiation of epithelial

cells, which are transformed (Behrens et al 1993). A more recent study observed that loss of E-cadherin lead to broad transcriptional changes associated with tissue remodelling which resulted in weaker cell-substrate adhesion and delayed migration in human mammary epithelial cells (Chen et al 2014). However, the study demonstrated that loss of E-cadherin alone was not enough to induce an EMT or enhance transforming potential of the epithelial cells. CAR has been shown to play a role in EMT and tumour progression. Cancer stem cells overexpressing CAR are more resistant to treatment with radiation and paclitaxel, self-renewing, and more tumorigenic than parental cells and silencing of CAR inhibits these functions (Zhang et al 2012). As previously described CAR controls E-cadherin recruitment to junctions (Morton et al 2013). Moreover, data in this thesis shows reduced localisation of E-cadherin in CAR overexpressing junctions (Fig.4.10). Perhaps, the interplay of CAR and FLNa, augmented by the loss of E-cadherin at junctions may drive EMT.

Importantly, CAR is emerging as a key player in the control of inflammation. Its expression is increased in rat cardiomyocytes in chronic autoimmune inflammatory conditions and in cystic fibrosis airway epithelial cells (Ito et al 2000; Sharma et al 2017). On the other hand, expression levels of CAR is reduced under pro-inflammatory environment induced by Alzheimer disease and/or systemic inflammation and also in primary cultures of murine hippocampal neurons and adult murine neural progenitor cells when incubated with inflammatory stimuli (Zussy et al 2016). Although these conflicting results suggest that tissue-specific role for CAR (refer to section 5.5) in controlling inflammation, they certainly highlight CAR's potential as an important therapeutic target in inflammatory disease. Recent

study in our lab showed that although CAR expression levels remain unchanged, it is hyper-phosphorylated *in vivo* in both acute and chronic lung inflammation models (Morton et al 2016). My data demonstrates that FLNa preferentially binds to phosphorylated CAR. Although FLNa has not been shown to have a direct role in transmigration of immune cells, FLNb, which is strongly homologous to FLNa, mediates ICAM-1 induced transmigration of leukocytes in the endothelium (van der Flier, & Sonnenberg 2001; Kanters et al 2008). It aids in the recruitment and lateral mobility of ICAM-1, and firm adhesion of the leukocytes to the endothelium (Kanters et al 2008). Moreover, my study has clearly established an increased activation of β1 integrin, which is the major integrin implicated in lung inflammation, promoted by CAR-FLNa binding (Sheppard 2003; reviewed in Teoh et al 2016). It is certainly worth exploring the role of FLNa in assisting CAR during transmigration of immune cells in the lung epithelium, particularly in promoting its lateral mobility and adhesion to JAM-L expressed on leukocytes. Such a study can also shed light on the role of CAR and the mechanism by which it *trans* heterodimerises with JAM family proteins as seen in the interactions between CAR expressed on skin epithelial cells and JAM-L on γδ T cells, and CAR on gut epithelial cells and JAM-L on neutrophils (Verdino et al 2010; Zen et al 2005).

5.4. ***Structure and spatial distribution of CAR regulates its biological function***

CAR is known to be differential expressed in an organ and time-specific manner. It is expressed in the brain, heart, lung, liver, testis, pancreas and kidney where it is localised to cell adhesive structures but performs potentially different roles within each tissue (Matthaus et al 2016). CAR has been suggested to play a key role in cardiomyocyte function and heart development and is essential in the development

of lymphatic vessels during embryonic stage but is rapidly downregulated after birth in this tissue (Asher et al 2005; Mirza et al 2012). In adults, CAR is involved in inflammatory response in pancreas and lungs (Hodik et al 2016; Ortiz-Zapater et al 2017). Our lab previously demonstrated that CAR plays a key role in lung epithelial cells in controlling inflammatory cell infiltration in vivo (Morton et al 2016). E-cadherin and other cell junction proteins stabilise and maintain the barrier function of lung epithelial cells, which is crucial to the integrity of airway epithelium. When triggered with inflammatory stimulus junction proteins including E-Cadherin, ZO-1 and JAM proteins, are down regulated and internalised (de Boer et al 2008, Nawijn et al 2011; Luissint et al 2014; Nawijn et al 2015). Our previous data has shown that CAR phosphorylated at threonine290/serine293 sites controls the stability and recruitment of E-Cadherin at junctions (Morton et al 2013). Moreover, TNFα, a pleotropic cytokine that triggers inflammatory response, also triggers phosphorylation of CAR at the same phospho-sites thereby reinforcing its ability to maintain stable junctions (Morton et al 2016). Interestingly, chronic exposure to TNFα generally leads to down regulation of CAR in other tissues but this is not seen in respiratory cells (Vincent et al 2004). It therefore seems that CAR's biological function and expression is tissue specific. It will be interesting to determine if the localisation and interactions between CAR and FLNa are different in other CAR-expressing cell types and whether this correlates with the function of CAR as an adhesion protein.

CAR expression in adult murine heart is required for normal atrioventricular-node conduction and normal cardiac function, as well as viral myocarditis (Lim et al 2008; Shi et al 2009). While CAR conditional knockout adult mice completely blocked virus

entry and the associated pathology including contractile dysfunction, these mice developed other pathological conditions including dilated intestinal tract, atrophy of the exocrine pancreas, complete atrio-ventricular block and abnormal thymopoiesis (Pazirandeh et al 2011). CAR is also expressed in axon tracks of the adult brain, sometimes at the presynapse in mature neurons, and is recruited to activated presynapses. Neurogenesis, synaptic function, and behaviour are severely affected by the genetic depletion of CAR in mouse (Zussy et al 2016). These findings further underline the role of CAR in tissue-specific manner and its importance in regulating cell:cell interactions in different cell types. Taken together with its ability to dynamically remodel cell:cell contacts (refer to section 5.2), CAR has the potential to play a key regulatory role in mediating dynamic cell adhesion processes during organ development and homeostasis.

Although the JAM family members have similar features, CAR, JAM4 and ESAM are structurally and functionally different in comparison to JAM-A,-B and –C. The cytotails of CAR, JAM4 and ESAM are also much longer than that of other members (Ebnet et al 2004). It is therefore quite a remote possibility for the other JAM molecules to have the same *TARS* motif that is phosphorylated by PKC δ to promote FLNa binding (Fig.3.2). There may be other binding sequence that needs to be explored. Mass spectrometric analysis of protein coimmunoprecipitates from the interaction between phosphomemetic / non-phosphorylated mutant of CAR and FLNa may shed some light on both alternate binding sites as well as other regulators of this interaction. Based on the evidence so far, it appears that CAR-FLNa interaction is largely relevant only in cells that form cell:cell contact (refer to section 5.2). In cells such as fibroblasts that do not form cell:cell contacts, CAR functions

mainly as a viral receptor (Hidaka et al 1999). Filamin A is involved in mediating cell spreading and survival in close association with β1 integrin (Kim et al 2008). It is unlikely that CAR is involved in this partnership but the possibility of CAR mediating β1 integrin function through a separate unrelated pathway cannot be entirely ruled out.

5.5. ***CAR may have a role to play in epithelial mechanosensing***

There has been a significant amount of work in recent years demonstrating a clear role for F-actin, filamin and focal adhesion proteins in transducing mechanical signals from the surrounding matrix (Borghi et al 2012; Liu et al 2010; Miyake et al 2006; reviewed in Goldmann 2012, Kuo 2013 & Razinia et al 2012). Increasing evidence also suggests that cell:cell adhesion proteins actively control and organise cytoskeletal rearrangements during processes such as cell division, cell extrusion in response to stress, and cell intercalation (reviewed in Luo et al 2013). E-cadherin at cell junctions is one of the recognised components of biomechanical signalling pathways, involved in mechanosensing and transmission of stress to regulate execution of these functional endpoints (le Duc 2010; Borghi et al 2012). Given the emerging similarities between CAR and E-Cadherin in terms of cross-membrane homo-dimerisation and cytoskeletal linkage, it seems entirely possible that CAR may perform a similar function and transmit forces to activate distinct intracellular pathways. The experiments in this thesis demonstrated that CAR did not appear to modulate epithelial cell responses to altered stiffness of the underlying ECM. However, as CAR would not be directly engaged with the altered substrate in those experiments, this does not rule out possibility that this force transmission only occurs when CAR is homodimerised across a junction. To study the force

transmission across CAR *trans* homodimers would require the isolation and immobilisation of CAR on substrates similar to method used to generate E-cadherin substratum (Drees et al 2005). It involves the generation of a recombinant CAR-Fc chimera with the CAR ectodomain fused at its C-terminus to the Fc domain of human IgG1. The recombinant CAR-Fc protein can then be bound to a chemical bridge of biotin-neutravidin-biotinylated protein A immobilised on a silanized glass cover slip. This method allows the fabrication of a substratum exposing the ectodomain of CAR in the correct orientation to be facilitate recognition by and binding of CAR on epithelial cells seeded on it. Immobilising the CAR-Fc on the surface of PDMS gels of differing stiffness would provide an interesting model system to explore the possibility of CAR's role in mechanotransduction in more detail in future.

5.6. **Conclusion**

This study presents filamin A as a novel binding partner of the cell adhesion receptor, CAR. It describes a role for CAR as a molecular switch in regulating integrin activation through its interaction with FLNa. It also explores the role of CAR in maintaining cell:cell adhesion stability which is crucial to maintaining an intact epithelium and throws light on the potential roles of CAR in epithelial cell spreading, mechanosensing and wound healing. CAR may, therefore, be an ideal candidate to facilitate the cross talk between cell:cell and cell:matrix adhesive structures via the cytoskeletal network under homeostatic and inflammatory conditions in the lung.

6. *Future Work*

This study raises a number of questions, which are crucial would be very important to follow up on in future to define how CAR is recruited and regulated at junctions, and how in turn it regulates its binding partners to control cellular events.

6.1. *Mechanism of FLNa binding to CAR*

Although this study shows that CAR and FLNa are part of the same complex, it does not show a direct binding between the two proteins. It has also been interestingly noted that CAR-FLNa colocalise mainly at cell junctions and highly concentrated at tricellular junctions as shown in Fig.6.1. Yeast two-hybrid experiment followed by FRET-FLIM analysis can be used to establish the direct interaction of the two proteins.

It has been established beyond doubt that integrin reactivation is a result of CAR-FLNa interaction. However, it still remains to be seen if CAR and integrin competitively bind to the same site of FLNa (as CAR binds albeit with lower affinity to the Rod2 domain which comprises Ig repeat 21 to which integrin binds) or have different binding sites. In case of the latter, it is unclear if CAR binding to FLNa involves the physical displacement of FLNa from sites of integrin-based focal adhesion or does CAR-FLNa binding structurally change sites of integrin binding on FLNa (i.e. does immunoglobulin repeat 20 mask integrin binding repeat 21?). Pulldown assays of CAR from lung epithelial cells using GST-tagged Ig repeats of FLNa will establish if CAR has an affinity for this region. If this is the case, a competitive binding assay between CAR and integrin can be carried where using GFP trap CAR-GFP and FLNa are allowed to co-precipitate in the presence of

increasing concentrations of purified integrin. This would shed light on the affinities of the two proteins for FLNa.

6.2. *Role of F-actin in the CAR-FLNa complex formation*

This study has shown that the stability of CAR-FLNa complex does not require F-actin. However, CAR has been shown to associate with F-actin (Huang et al 2007) and binds strongest to the Actin Binding Domain of FLNa. It is likely that the trio form a complex. One possibility is that F-actin may be acting as a scaffold, binding individually to CAR and FLNa, thus enabling an interaction between them. The other is FLNa may be acting as the linker between CAR and F-actin thereby integrating cell junction dynamics with the cell architecture. Co- sedimentation assay of F-actin with CAR in lung epithelial cells will reveal the relationship between the two proteins. Super resolution imaging of CAR, FLNa and F-actin over time at junctions that are assembling (as described in section 2.2.15) or dissembling (this can be achieved by washing out Ca^{++} in the growth media or by adding mild doses of actin depolymerising agents as described in section 2.2.12) will add clarity to their distribution and recruitment patterns.

6.3. *Upstream regulation of CAR-FLNa binding*

PKC δ is the most likely candidate to drive the CAR-FLNa interaction as discussed in the previous section. Co-precipitating CAR and FLNa from lung epithelial cells knocked down for PKC δ would confirm its role in controlling this interaction.

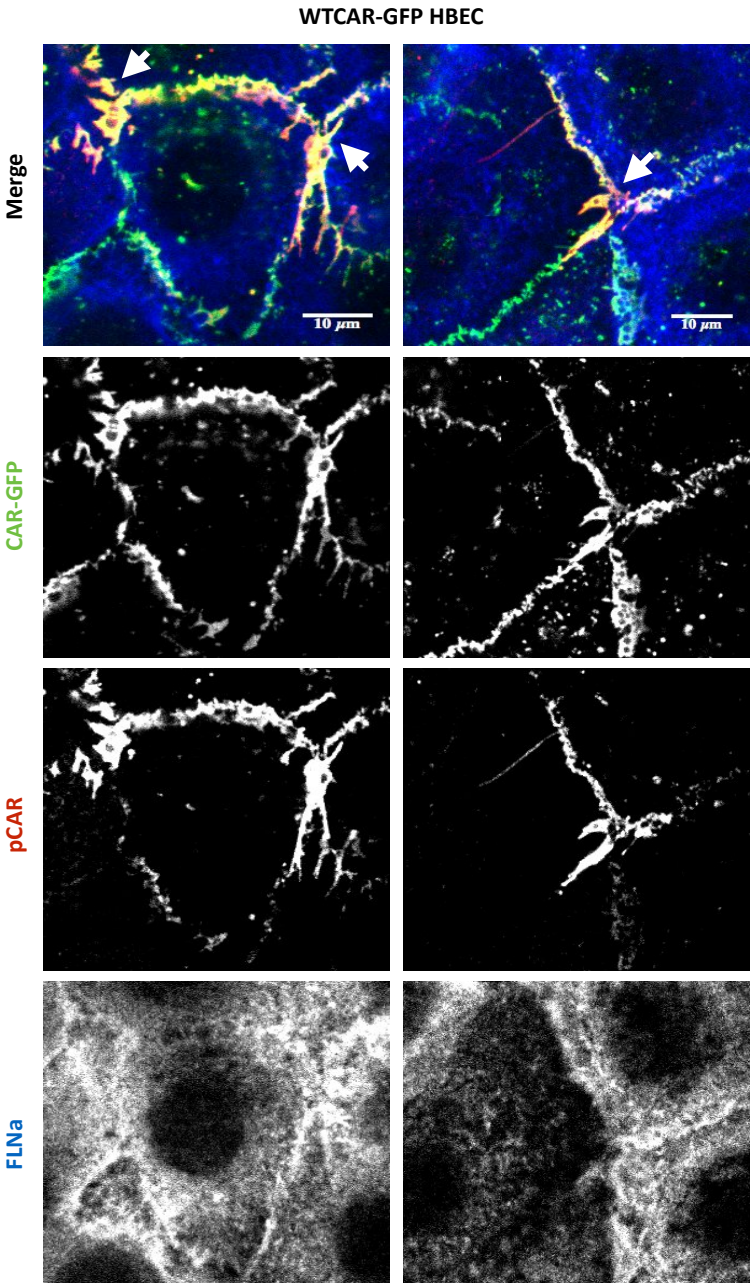


Fig.6.1. **pCAR and FLNa are enriched at tricellular junctions of epithelial cells.** (A) Confocal images of monolayers of WTCAR HBECs over-expressing GFP-tagged WT-CAR grown on collagen in calcium-containing media and immunostained for FLNa (blue) and pCAR (red). White arrows point at tricellular junctions. Scale bar : 10μm

6.4. *CAR as a mechanosensor*

Recombinant CAR-Fc chimera immobilised on compliant matrices of varying stiffness can be used as substratum to promote CAR:CAR interactions. Z-stacks obtained on confocal microscopes at the cell:matrix interface can be used to analyse the role of CAR in mechanosensing and mechanotransduction (refer to section 5.6. for details of the technique).

The enrichment of CAR at sites of high tension, i.e. tricellular junctions, is an interesting observation. Generating a calibrated biosensor to measure the tension across CAR as shown by single molecule fluorescence force spectroscopy (Hohng et al 2010) would reveal CAR's mechanosensing role, if any.

6.5. *Effect of CAR-FLNa complex formation in 3D environments and in vivo*

Cell behaviour in 2D and 3D environment do not always correlate. Culturing cells using magnetic levitation to create 3D rings of cells that close over time can be used to test the viability and migratory patterns in 3D (as described in Timm et al 2013). The next logical step would be to assess CAR-FLNa colocalisation from samples of human lung tissue or murine models with induced lung inflammation. The CAR-knockout murine model may also be used to determine wound healing *in vivo*.

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